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RELATIONSHIP OF ASCHOFF BODIES IN CARDIAC ATRIAL APPENDAGES TO THE NATURAL HISTORY OF RHEUMATIC HEART DISEASE

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The purpose of this study was to investigate the relationship between the pre-operative clinical course of patients with rheumatic heart disease, who underwent commissurotomy of the mitral valve, and the occurrence of Aschoff bodies in the left atrial appendage removed at the time of operation. With the development of surgical intervention in rheumatic heart disease, considerable discussion has arisen concerning the significance of the Aschoff bodies frequently found in atrial appendages removed at operation.[1-27] The patients selected for mitral commissurotomy were, in the great majority, considered free of rheumatic activity at the time of operation, often after extensive and careful clinical examination. It appeared anomalous, therefore, that the incidence of Aschoff bodies in atrial appendages has been reported to be as great as 74 per cent.[4] Efforts to explain this apparent contradiction have thus far been disappointing. Moreover, the occurrence of Aschoff bodies has not generally correlated with electrocardiographic abnormalities, elevation of the antistreptolysin O titer, rapid erythrocyte sedimentation rate, or the appearance of C-reactive protein,[5,14,18,23] nor has the presence of Aschoff bodies in atrial appendages appeared to be of prognostic significance.[25,28] These discrepancies have led some students of rheumatic heart disease to conclude that atrial Aschoff bodies are not indicative of rheumatic activ-

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ity,[11] although it has been found repeatedly that the appearance of Aschoff bodies in atrial appendages correlates closely with their occurrence in the ventricular myocardium.[9,10,12,17,26,29] Other investigators have attempted to resolve this problem by considering atrial Aschoff bodies to be indicative of rheumatic activity only if they are accompanied by certain connective tissue alterations and exudative inflammatory reactions.[24] If the morphologic criteria and interpretation employed by the latter investigators were valid, the incidence of rheumatic activity at the time of mitral commissurotomy in a large group of patients studied by them would be only about 2 per cent.

Most patients who submit to mitral commissurotomy do so because their symptoms of cardiac disease are progressively worsening. Indeed, the most suitable candidates for surgical intervention are found among the patients with progressive disability.[28,30,31] This raises the question: Why does the cardiac status of these patients deteriorate? It appears to us that there are two probable explanations.[32–34] One is further embarrassment of the patient's marginally compensated heart by increased physiologic demands associated with such conditions as anemia, severe infection, emphysema and chronic bronchitis, pregnancy, or thyrotoxicosis; or further embarrassment by the effects of another superimposed cardiovascular disorder such as hypertensive or arteriosclerotic disease, thrombo-embolism, pulmonary arteriosclerosis, or renal disease. The other cause of progressive cardiac decompensation is rheumatic activity itself. If this hypothesis is valid, and if Aschoff bodies are manifestations of active rheumatic heart disease, then patients with rheumatic heart disease, who are free of nonrheumatic cardiovascular disorder and other conditions, such as those given above, would show a meaningful correlation between the preoperative clinical course and findings in their atrial appendages. More specifically, Aschoff bodies would be found with greater incidence among patients showing recent, marked, progressive worsening, as compared with those who had no recent change in their cardiac status.

Material and Methods

The records of The New York Hospital list 103 patients under the age of 41 years who underwent mitral commissurotomy for rheumatic heart disease before 1957. Histories in these cases had been recorded in detail by at least 3 observers on each admission. Only patients below the age of 41 years were selected for this investigation in order to minimize the effect of nonrheumatic arteriosclerotic cardiovascular disease. Only those patients who underwent mitral commissurotomy before 1957 were studied to provide for a period of 2½ years of postoperative follow-up. Two patients were excluded because their clinical histories were inadequate. Fifteen additional cases were eliminated because of the following conditions: 11 patients were pregnant at the time of operation; 1 patient had experienced repeated episodes of pulmonary

embolization; 1 had hypertension; 1 had diabetes mellitus; and 1 was considered to have Lutembacher's syndrome.

Paraffin blocks of the atrial appendages were obtained from the laboratory of Surgical Pathology through the courtesy of Dr. John M. Pearce. New sections were cut and stained with the hematoxylin and eosin, Masson trichrome, and phosphotungstic acid-hematoxylin stains. In addition, the original sections stained with hematoxylin and eosin were examined. The amount of tissue was inadequate in 5 cases and they, too, were excluded.

The final sample consisted of tissue from 81 patients, 64 women and 17 men, ranging from 20 to 40 years of age.

All atrial appendage sections were first independently examined by both authors and later reviewed together. This material was classified into two groups, one with and the other without specific rheumatic lesions of the Aschoff body type. The criteria used for the recognition of these lesions were based on the results of long-term studies recently reported by one of the authors.[27] In atrial appendages rheumatic lesions of Aschoff body type appear to us to be of two kinds:

(1) Aschoff bodies that originate from rheumatic injury to striated heart muscle cells (Figs. 1,3,4 and 6). As it expands, this body, developing from heart muscle cells just beneath the subendocardial zone of smooth muscle cells and connective tissue, sometimes thrusts its way toward the endocardium so that part of it comes to lie in the subendocardium. In those areas where the subendocardial zone is thin, these bodies, in expanding, come to lie close to the overlying endocardium and may be interpreted erroneously as evidence of endocarditis (Figs. 1 and 6).

(2) Lesions, generally referred to as Aschoff bodies, that originate from rheumatic injury to smooth muscle cells (Fig. 5) in the subendocardium. These lesions lie entirely in the subendocardium and can mimic, sometimes very closely, Aschoff bodies derived from striated heart muscle cells.

In this communication the term Aschoff body, rather than lesion of Aschoff body type, will be used to refer to the specific rheumatic lesions both of striated myocardial elements and of smooth muscle cells in the subendocardial tissue. The final histologic diagnoses were arrived at without knowledge of the corresponding clinical records.

The clinical records were likewise analyzed and classified independently by both authors and later reviewed together. In examining the clinical data, particular attention was paid to the time of onset and to the progression of cardiac symptoms prior to operation. Emphasis was placed on such symptoms as increasing exertional dyspnea, orthopnea, paroxysmal nocturnal dyspnea, episodes of hemoptysis and progressive fatigue. The patients were classified into 3 major groups. Group I consisted of those who showed steady progression of cardiac symptoms with evidence of progressive reduction of cardiac reserve for 18 months or less prior to operation. Eleven of these had been entirely asymptomatic before their pre-operative deterioration. Group II comprised those patients who had shown steadily progressive worsening of cardiac symptoms and evidence of decreasing cardiac reserve for a period longer than 18 months prior to operation. Group III comprised patients who also had severe rheumatic heart disease but had shown no evident deterioration of their cardiac status during the 2 years prior to operation.

The patients showing deterioration prior to operation (Groups I and II) were further divided into subgroups "a" and "b" depending upon the degree of worsening. Those who had experienced well documented, marked deterioration were classified in subgroup "a," while those whose deterioration was less marked, and sometimes evaluated with difficulty, were classified in subgroup "b." These subdivisions brought the total number of clinical groups to 5 (Ia, Ib, IIa, IIb, and III). Examination of the clinical records and decisions as to clinical grouping were made without knowledge of the findings in the atrial appendages.

Results

Histologic Observations

Of the entire group of 81 atrial appendages examined 51, or 63 per cent, contained Aschoff bodies. Three other appendages showed numerous small foci of necrosis of heart muscle cells with nucleated and nonnucleated myofiber fragments and plasma cells in apparent reaction to the necrosis (Fig. 2). These 3 cases were classified in clinical groups Ia, Ib, and III, respectively. The significance of these nonspecific myocardial lesions is not clear, but they appear to us to be an expression of active rheumatic disease of the myocardium.[27] Necropsy examinations were performed on 2 patients in this study who died shortly after commissurotomy; one of these died in the operating room and the other on the ninth postoperative day. In one case, Aschoff bodies were present in the myocardium of the left ventricle as well as in the atrial appendage. In the other case, foci of necrosis of heart muscle cells comprising myofiber fragments and plasma cells were found in the atrial appendage. Similar lesions were found in the left ventricle at necropsy, but no Aschoff bodies were encountered.

Table I

OCCURRENCE OF ASCHOFF BODIES IN ATRIAL APPENDAGES IN RELATION TO DURATION AND SEVERITY OF PRE-OPERATIVE DETERIORATION OF CARDIAC STATUS

Clinical group based on progression of symptoms prior to commissurotomy			Aschoff bodies		Age of patients in groups		Normal sinus rhythm (%)
			No. with bodies *				
Group	Duration of progression	Severity of progression	No. in group	%	Average	Median	
Ia	18 mo. or less	Marked, definite	19/20	95.0	30.9	31	90.0
Ib	18 mo. or less	Minimal, indefinite	10/15	66.7	34.4	36	46.6
IIa	Longer than 18 mo.	Marked, definite	13/17	76.5	32.0	32	70.6
IIb	Longer than 18 mo.	Minimal, indefinite	5/16	31.3	34.4	35	43.7
III	No progression of symptoms for at least 2 yr.		5/13	38.5	31.7	31	38.5
Total			51/81	63.0			

* Numerator represents the number of patients in whom Aschoff bodies were found; the denominator the number of patients in group.

Statistical analysis[28] of the incidence of Aschoff bodies in the various clinical groups:

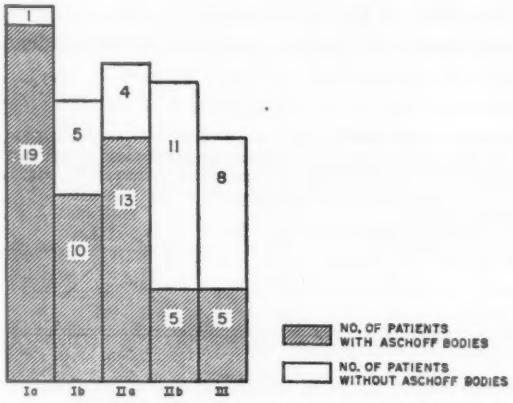
Groups Ia + IIa compared with Groups Ib + IIb + III $X^2_{(1)} = 12.8$; $0.001 > p > 0.0001$

Groups Ia + IIa compared with Group III $X^2_{(1)} = 9.0$; $0.01 > p > 0.001$

Group Ia compared with Group III $X^2_{(1)} = 10.3$; $0.01 > p > 0.001$

Correlation Between Histologic Observations and the Preoperative Clinical Histories

The results are shown in Table I. The most significant groups for comparison are group III, comprising those patients with a clinically stationary cardiac status, and groups Ia and IIa, comprising those patients with marked, steady progression of cardiac symptoms up to the



TEXT-FIGURE 1. Clinical groups.

time of operation. It should be noted that Aschoff bodies were found in 38.5 per cent of patients in group III. In contrast, Aschoff bodies were found in 86.5 per cent of the patients showing marked progression of cardiac symptoms prior to operation (Ia and IIa combined) and in 95 per cent of those with marked progression that began only 18 months or less prior to operation (Ia). The only patient (Fig. 2) in group Ia in whom Aschoff bodies were not found was one of the previously mentioned 3 cases with numerous small foci of necrosis of heart muscle cells.

Correlation Between Cardiac Rhythm and the Occurrence of Aschoff Bodies

It has been shown by others[14] that among the patients, taken as a group, with normal sinus rhythm prior to operation, there was a higher incidence of Aschoff bodies in the atrial appendages than among those with atrial fibrillation. In the present study (Table I) the highest incidence of normal sinus rhythm and the highest incidence of Aschoff bodies occurred among the patients in group Ia. This group was composed of patients whose cardiac status had deteriorated markedly over a relatively short period of time from a state of comparative good health to

severe illness. Eleven of these 20 patients were free of cardiovascular symptoms 1½ years or less prior to operation. A similar association is found in Table II, where it is seen that among patients who had severe cardiovascular symptoms for only one year or less, there is a much lower incidence of atrial fibrillation than among those chronically ill.

Incidence of Aschoff Bodies in Various Age Groups

The patients were also classified in 5-year age groups. The oldest patients were 40 years of age by selection in this study. Those in the youngest age group (20 to 25 years) had the highest incidence of Aschoff

TABLE II

OCCURRENCE OF ATRIAL FIBRILLATION AT THE TIME OF OPERATION IN RELATION TO THE DURATION OF SEVERE SYMPTOMS OF RHEUMATIC HEART DISEASE

Duration of severe symptoms in years prior to commissurotomy	Normal sinus rhythm (%)	Atrial fibrillation (%)	No. of patients in group
0 to 1 year	93	7	14
1 to 5 years	68	32	25
Greater than 5 years	45	55	42

bodies (91.6 per cent); while in the other age groups the incidence was about the same (52.6 per cent, 66.7 per cent, and 55.2 per cent, respectively). The average and median age of the patients in the 5 clinical groups was calculated and found to be essentially similar (Table I). It was thus shown that age was not responsible for the difference in incidence of Aschoff bodies among these clinical groups.

CONCLUSIONS AND SUMMARY

A significant relationship has been demonstrated between the preoperative clinical course of patients with rheumatic heart disease and the occurrence of Aschoff bodies in their left atrial appendages. Of those patients who had experienced marked, progressive worsening of cardiac symptoms for 18 months or less prior to operation, 95 per cent showed Aschoff bodies as compared with 38.5 per cent in patients whose severe cardiac symptoms had remained stationary for at least 2 years prior to operation (Table I). These data strongly indicate that Aschoff bodies in themselves are diagnostic of active rheumatic heart disease.

In accord with the observations of others,[14] it has been found that among patients, taken as a group, with normal sinus rhythm prior to operation there is a considerably higher incidence of Aschoff bodies in atrial appendages than among those with atrial fibrillation. The data given in Table II show how atrial fibrillation is, in general, a reflection

of the chronicity of severe rheumatic heart disease. Of those patients who underwent mitral commissurotomy because of recent, marked, progressive deterioration of cardiac status (group Ia) 90 per cent exhibited normal sinus rhythm and 95 per cent showed Aschoff bodies in the atrial appendages. These findings contrast with the much lower incidence of normal sinus rhythm (38.5 per cent) and of Aschoff bodies (38.5 per cent) in the patients with severe, long-standing rheumatic heart disease but with stationary cardiac status for at least 2 years prior to operation (group III).

It appears that rheumatic heart disease is sometimes a chronic, smoldering process with long episodes of remission and exacerbation. Many episodes of rheumatic activity do not produce the clinical manifestations classically considered to be characteristic of an attack of rheumatic fever. And many of these episodes must be clinically mute, in view of the large proportion of patients severely handicapped with rheumatic heart disease who give no history of experiencing an attack of acute rheumatic fever or chorea. This is further borne out by our observation of specific histologic evidence of active rheumatic heart disease in 38.5 per cent of patients with severe rheumatic heart damage who had no evident deterioration in the cardiac status during the 2 years prior to commissurotomy (clinical group III). However, other findings here reported indicate that when subclinical episodes of rheumatic activity occur in patients with severe cardiac damage from previous rheumatic disease, they often manifest themselves by progressive worsening of cardiovascular symptoms (clinical groups I and II). Indeed, it appears to us that the chief cause of cardiac decompensation in patients with rheumatic heart disease, at least up through the age group here studied, is rheumatic activity itself.

Although Aschoff bodies are diagnostic of active rheumatic heart disease, their presence in itself is not prognostic of future events in the natural history of the disease. If a patient has active rheumatic disease on the day of commissurotomy, it does not necessarily follow that this particular episode of activity will long continue and lead to recurrent decompensation with progressive myocardial alterations or new valvular alterations. Instead, the surgeon may fracture the patient's mitral valve near the end of a phase of rheumatic activity. In such an instance one would observe marked improvement postoperatively, due not only to improved hemodynamics related to commissurotomy, but also to improved function of the myocardium resulting from remission of activity. By the same token, absence of rheumatic activity at the time of operation does not preclude subsequent reactivation associated in many cases with worsening of myocardial status.

In explaining improvement or retrogression of patients with rheumatic heart disease, one must bear in mind that the natural history of this disease is often characterized by alternating periods of remission and activity of varying duration and severity. Aschoff bodies are an expression of this activity.

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LEGENDS FOR FIGURES

Photographs were prepared from sections stained with hematoxylin and eosin.

FIG. 1. Two Aschoff bodies among many found in the deep subendocardial tissue and contiguous myocardium of the atrial appendage. Mono-, multi-, and non-nucleated basophilic elements are derived from heart muscle fibers subjacent to the subendocardium. × 282.

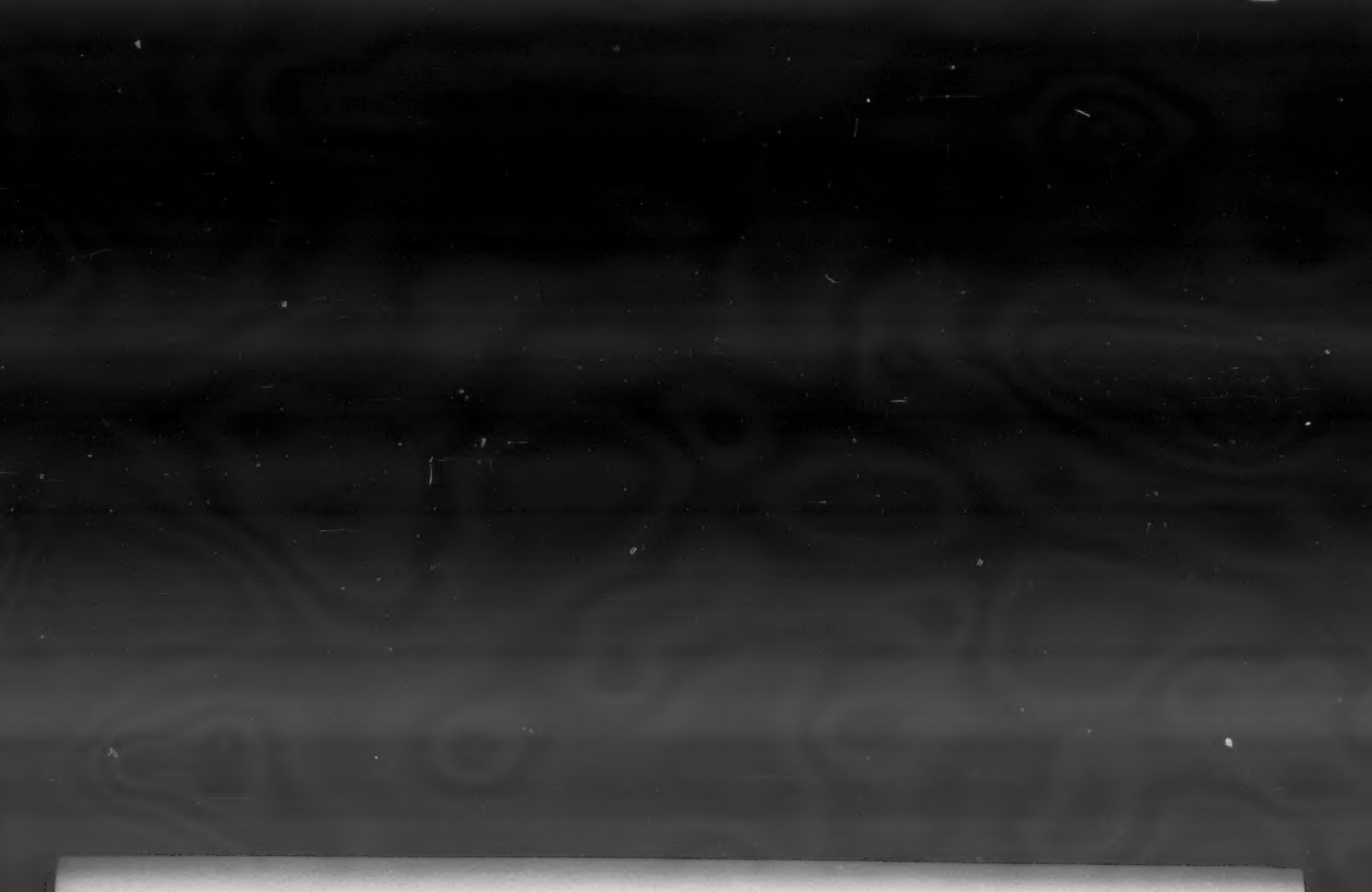
Case Report. Clinical group Ia. A 37-year-old white woman had rheumatic fever 19 years prior to operation, but was free of symptoms of cardiac disease until 7 months prior to commissurotomy. She then developed hemoptysis, dyspnea on climbing 2 flights of stairs, and fatigue. Symptoms progressed steadily, and 1 month prior to operation she noted chest pain, cough, and marked shortness of breath. Two weeks later there was an episode of epistaxis and, after several days, pain in one elbow and hand. At the time of operation there was normal sinus rhythm. Marked mitral stenosis was found; no regurgitation was evident. Commissurotomy was considered very satisfactory. The patient made an uneventful recovery and has remained markedly improved for 5 years.

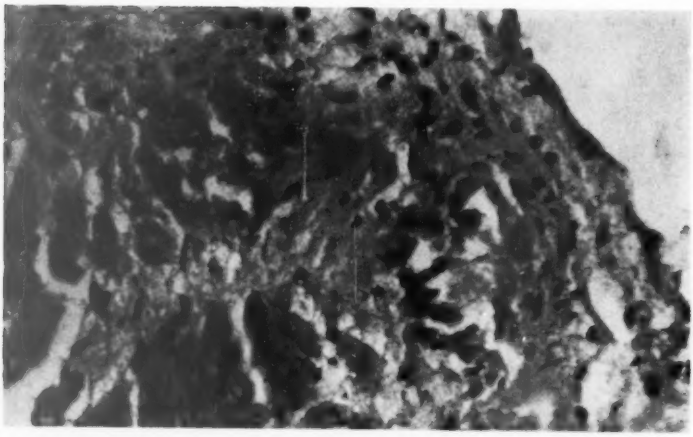
FIG. 2. The atrial myocardial alteration comprises disintegrating cardiac myofibers. Some fragments are multinucleated; others show either one or no nuclei. Numerous small cells appear in the main to be plasma cells. Such focal lesions were widespread throughout the myocardium of the atrial appendage. × 282.

Case Report. Clinical group Ia. A 39-year-old white man had 2 attacks of chorea 22 and 24 years prior to operation. He was free of symptoms of cardiac disease until 9 months before operation when he experienced episodes of dyspnea and hemoptysis. His symptoms increased steadily, and by the time of operation he was dyspneic after walking 1 block and had atrial fibrillation. The erythrocyte sedimentation rate ranged from 4 to 11 mm./1 hr. Marked mitral stenosis was associated with a small amount of regurgitation. Commissurotomy was considered to be satisfactory. Marked postoperative improvement for 2 years was followed by progressive deterioration of the cardiac status, resulting in death 4 years after operation. At necropsy, focal lesions, like those at biopsy (Fig. 2), were found throughout the myocardium. Sections were supplied by Dr. George Sharnoff.

FIG. 3. Aschoff bodies in the myocardium of the atrial appendage. These lesions are characterized by mono-, multi-, and non-nucleated basophilic elements, some arranged in rows as in the left portion of the picture. Only a few more of these lesions were found. × 113.

Case Report. Clinical group Ib. A 24-year-old Negro woman had rheumatic fever 17 years before operation. She first developed congestive heart failure 7 years prior to operation, during her first pregnancy. Following delivery, she noted progression of symptoms, with considerable exertional dyspnea and anasarca. On treatment with mercuhydrin she improved, and her cardiac status remained approximately stationary until about one year prior to operation when there was progressive reduction of exercise tolerance. At the time of operation she had atrial fibrillation, and the erythrocyte sedimentation rate was 3 mm./1 hr. Marked mitral insufficiency was found and no commissurotomy was carried out. After a period of improvement of about 1 month she became progressively worse and finally died of heart disease 2¾ years postoperatively. No necropsy was performed.





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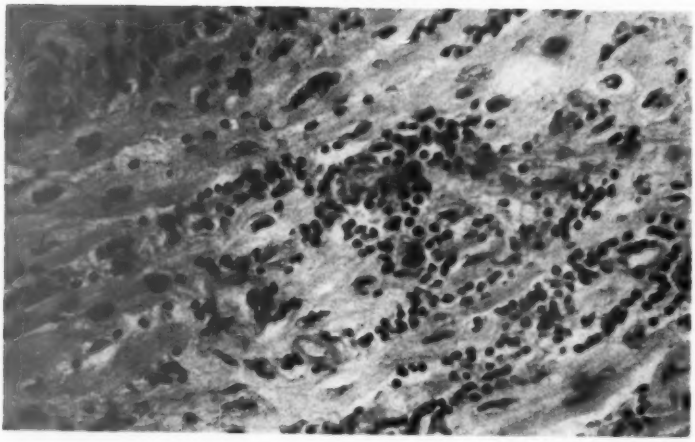

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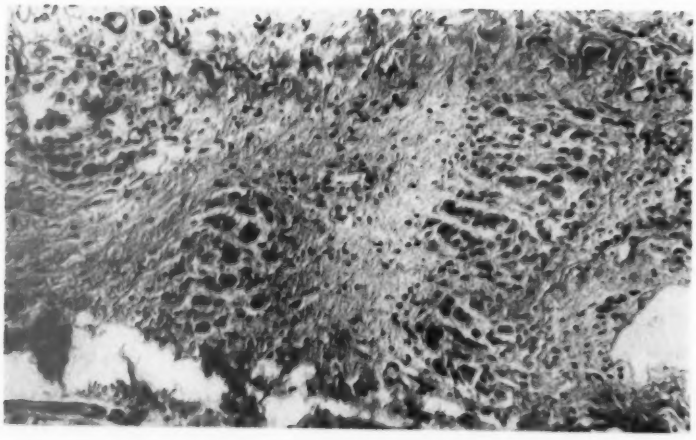

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FIG. 4. An Aschoff body, clearly derived from heart muscle cells, and containing mono-, multi-, and non-nucleated basophilic and amphophilic myogenic elements. Numerous such lesions were found in the deep subendocardial tissue and contiguous myocardium. × 282.

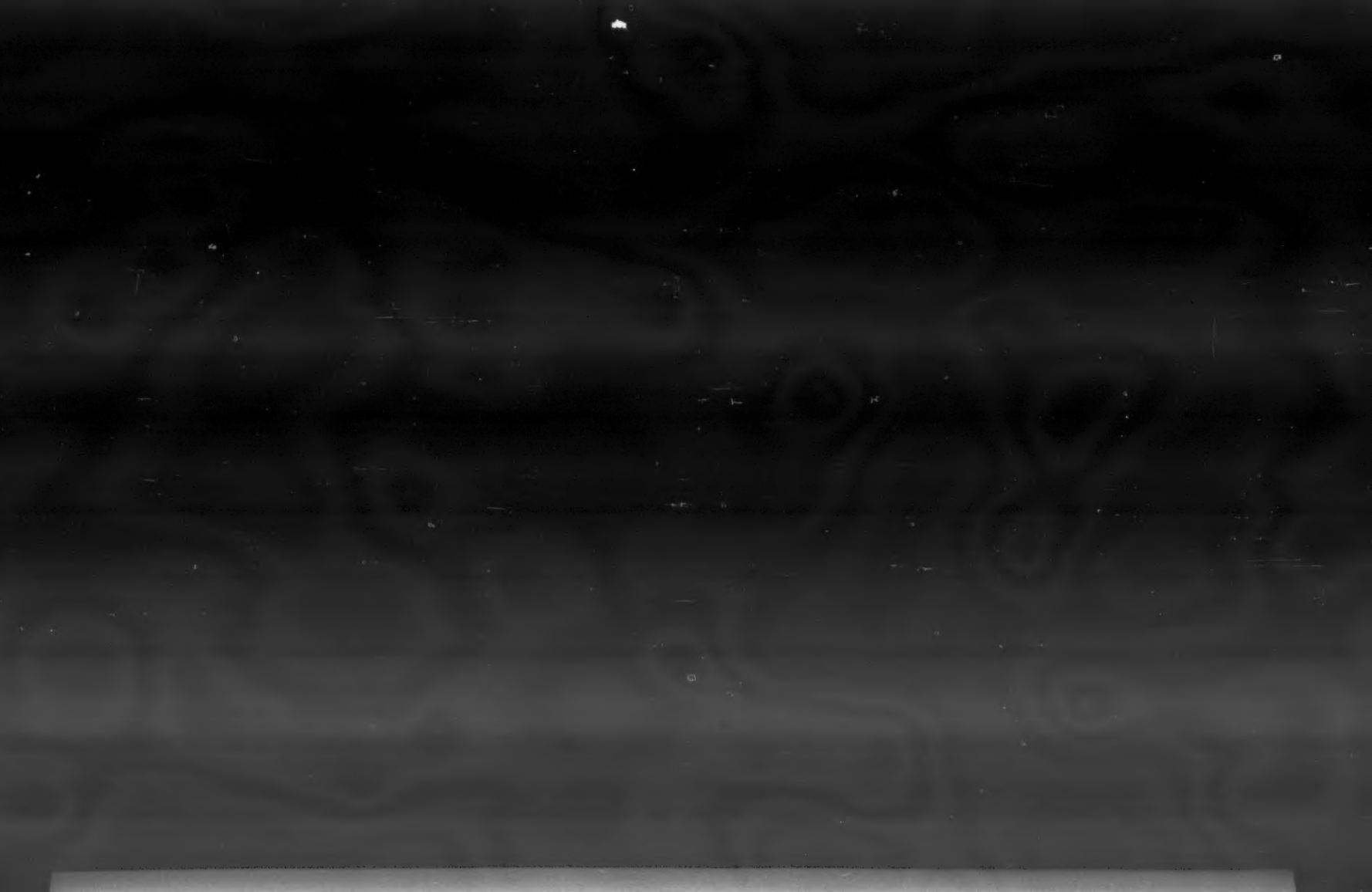
Case Report. Clinical group IIa. A 23-year-old white woman had no history of rheumatic fever but had experienced some shortness of breath and palpitation during the third month of her first pregnancy 5 years before operation. Her symptoms improved following a full-term delivery. Over the last 2 years prior to operation, the patient developed progressively worsening dyspnea and ankle edema. At the time of operation, she had atrial fibrillation, dyspnea after climbing a flight of stairs, and orthopnea requiring 2 pillows. The erythrocyte sedimentation rate was 4 mm./1 hr. At operation, marked mitral insufficiency as well as stenosis was found. A commissurotomy was performed. She experienced marked improvement for 1 year but then developed overt rheumatic fever, associated with massive cardiac enlargement, and died 2 years postoperatively. No necropsy was performed.

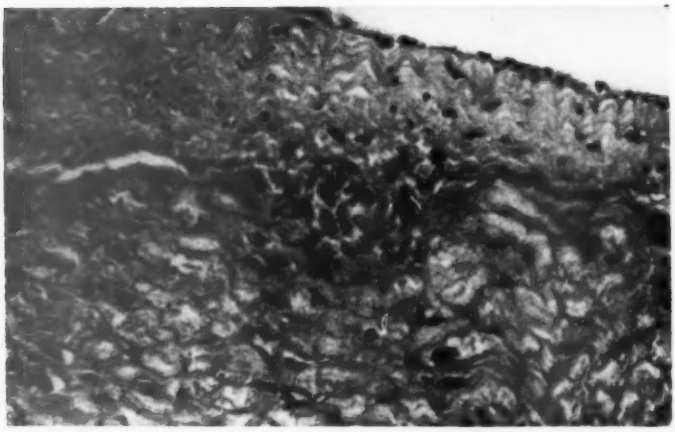
FIG. 5. Two lesions of Aschoff body type lie entirely within the subendocardial tissue. These contain mono-, multi-, and non-nucleated basophilic and amphophilic elements probably derived from smooth muscle cells. The lesions lie where groups of normal smooth muscle cells very probably existed originally, as indicated by the occurrence of an avenue of recognizable smooth muscle cells extending from the right and from the left of the illustrated lesions. Many such subendocardial lesions were found. × 282.

Case Report. Clinical group IIb. A 26-year-old white woman had experienced pains in her ankles 8 years before operation but was free of cardiac symptoms until 3½ years later when, during her second pregnancy, she developed dyspnea and ankle edema. Following full-term delivery, there was apparently very gradual worsening of dyspnea on exertion for the 4 years prior to operation. At the time of operation she had dyspnea after walking 6 blocks or climbing 2 flights of stairs, normal sinus rhythm, and the erythrocyte sedimentation rate was 13 mm./1 hr. A markedly stenosed mitral valve, associated with minimal regurgitation, was satisfactorily fractured. Moderate improvement occurred and has persisted for 5 years after operation.

FIG. 6. An Aschoff body, clearly derived from a bundle of heart muscle cells just beneath the subendocardial tissue contains mainly mono-, multi-, and non-nucleated basophilic elements. Numerous such lesions were found. × 282.

Case Report. Clinical group III. A 29-year-old white woman had rheumatic fever 17 years prior to operation. Since that time she had experienced marked fatigue and dyspnea after climbing a flight of stairs or walking a half block, and had been taking digitalis. Four years before operation she developed ascites and marked ankle edema. During the 2 to 3-year period preceding operation, her cardiac status remained stationary with 2 pillow orthopnea, anasarca, and dyspnea on walking 6 steps. At the time of operation, she had atrial fibrillation and the erythrocyte sedimentation rate was 12 mm./1 hr. At exploration she was found to have marked mitral insufficiency and a commissurotomy was not performed. The patient's condition failed steadily, and she died 2 years later. No necropsy was performed.





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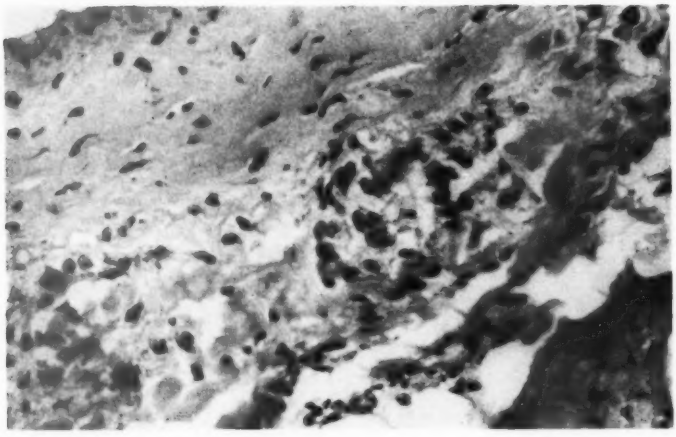

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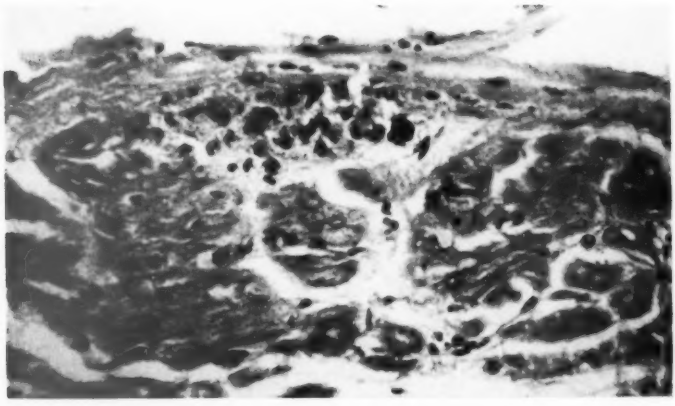

6

EXPERIMENTAL ARTERIOSCLEROSIS DUE TO HYPERVITAMINOSIS D

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Chronic progressive vascular disease resembling human athero-arteriosclerosis has not been produced in experimental animals. Fibrocalcific degenerative and reparative sequences similar to those occurring in the human disease may follow experimental arterial medial injury in animals.[1] Other sequences characterized by lipophage accumulations and atheromatous deposits resembling those in man may be induced by hyperlipemia and hypercholesteremia in animals.[2] Lesions which still more closely resemble those in man may be produced in animals by the simultaneous combination of local arterial medial injury, hypercholesteremia and hyperlipemia.[3,4]

These observations on locally induced lesions have indicated that experimental production of a generalized arterial medial disease combined with appropriate alterations in the lipid composition and dynamics of the blood might lead to a better understanding of the pathogenesis of human athero-arteriosclerosis.[5] It has been shown that generalized arterial medial disease follows the administration of excessive amounts of irradiated ergosterol or Vitamin D_2 in several animal species and man.[6-8] In undertaking the experimental plan outlined above, a systematic study of hypervitaminosis D in rabbits was carried out.[9] The present report is concerned principally with pathologic alterations found in the vascular system in hypervitaminosis D and comparison of these changes with those commonly found in human arteriosclerosis.

METHODS

Male albino rabbits from a common stock, about 5 pounds in weight and 3 months of age, were used. They were maintained on a standard Purina Rabbit Pellet diet supplemented with fresh vegetables.

The irradiated ergosterol was dissolved in peanut oil (15 mg. per ml.; supplied through the courtesy of Abbott Laboratories, North Chicago, Illinois). The Vitamin D potency of this solution was 10^6 U.S.P. units per ml. This was given intramuscularly in doses of 0.1 ml. (10^5 U.S.P. units) at daily, biweekly or triweekly intervals. The regime was varied so that minimal and maximal pathologic features could be defined and sequences in their evolution recognized. At first, the dosage was regulated at levels just below the quick lethal range. Pathologic changes developed rapidly, and

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most animals lost weight and died within 6 weeks. Later on, the regime was regulated to facilitate analysis of minimal, sublethal and early pathologic effects, so that animals were either sacrificed at an early stage or given a small dose and sacrificed at a later stage. Finally, the regime was modified so that rest periods of 2 or 3 weeks were alternated with dosage periods of 1 or 2 weeks. This allowed the development of chronic forms of the disorder, lasting several months.

Analyses for serum calcium, total phosphorus and inorganic phosphorus were done at intervals of 1 to 2 weeks.

At the end of each experiment a necropsy was done and a record made of the gross observations. Samples of all tissues and organs were fixed in formaldehyde (4 per cent, U.S.P.) and subsequently prepared for microscopic examination. Transverse sections were also made through the aortic arch, upper and lower abdominal aorta, common carotid artery, brachial artery, pulmonary artery, hilar pulmonary vessels, renal artery, common iliac artery, femoral artery and vessels at the hilus of the spleen, liver and kidney. Ninety-seven animals were useful for the purposes of this report, with studies of 82 additional animals on other dietary and viosterol regimes available for supplementary data. For control purposes, we made complete microscopic studies of 78 normal rabbits from the same stock, of the same age distribution and on the same diet.

Results

Histologic sequences in the walls of arteries

Adventitia

Involvement of the adventitia was uncommon. When lesions did occur, two independent sequences were recognized. The most important was essentially inflammatory. It began as an acute or subacute periarteritis or panarteritis of low intensity characterized initially by edema and nodose swelling of collagen in local areas.[9] These changes were accompanied or followed by a meager infiltration of the arterial wall and adventitia by heterophils and macrophages. The process ordinarily subsided in the media and intima without producing much structural damage but tended to persist in focal areas in the adventitia where collagen deteriorated in the midst of locally proliferating histiocytes. Occasionally, the proliferative reactions centered about degenerating cardiac or skeletal muscle cells adjacent to foci of adventitial inflammation. The deterioration of collagen, the histiocytic reaction and the incorporation of regional muscle cells in the periarterial response resembled alterations often encountered in acute rheumatic myocarditis in man.[9] The arteritis, with rare exceptions, was encountered only around vessels in cardiac and skeletal muscle. Extramuscular, small arterial branches and large arteries leading to muscle were not involved.

The second type of lesion in the adventitia was a vascularizing stromal reaction (Figs. 4 and 8). This was secondary to medial degeneration and occurred especially when degenerative-calcific changes in the media extended to the adventitia. These changes stimulated stromal penetration

and early vascularization of the media by adventitial elements which grew in and around the degenerated calcified zones. Thereafter, there was fibrosis of the media and slow resorption of medial calcific deposits with occasional foci of osteogenesis. This type of reaction was confined principally to the external third of the aortic media and the degenerated calcified media of the iliac and femoral arteries (Figs. 4 and 8).

External Elastic Membrane

The external elastic membrane of most arteries was usually uninvolved even though the adjacent adventitia and media showed lesions (Fig. 7). At times, however, the membrane was lightly basophilic. This alteration predisposed to mineralization, followed by transverse fragmentation of the membrane and stromal ingrowth into the media from the adventitia (Fig. 8).

The renal artery was the only vessel which showed conspicuous elective lesions in the external elastic membrane, but, even in this instance, the internal elastic membrane and media were always severely affected. There was evidence that preservation of anatomic continuity and structural integrity of the external elastic membrane in the presence of severe medial disease was a deterrent to stromal penetration and vascularization of the media by activated adventitial elements.

Media

The patterns of involvement of the media varied from one artery to another. In general, however, they were similar in the walls of arteries with the same dimensions and structure.

Elastic Arteries. The elastic arteries showed two forms of medial alteration. The common form began with swelling of the spaces between the innermost elastic membranes (Fig. 5). These spaces, presumably swollen from an increment of ground substance, were traversed by a delicate meshwork of barely recognizable fibrils. Fine granules of calcium were deposited on these fibrils and in the ground substance between them (Figs. 3 to 6). The deposit was more conspicuous where fibrils were condensed along and in elastic lamellas. It appeared, therefore, as though the elastic lamellas were impregnated with calcium on their surfaces (Fig. 3). Close inspection, however, disclosed a delicate, intrinsic fibrillar pattern as a locus for calcium deposition throughout many apparently solid elastic membranes (Fig. 4). As calcium accumulated in these locations, the intervening fibrocytes and smooth muscle cells were either undergoing degeneration or being replaced by large pale cells with hyperchromatic nuclei resembling hypertrophied, immature smooth muscle elements (Fig. 5). The degeneration of cells was

closely related spatially and temporally to that of calcium deposition, and cell regeneration was stimulated only when the deposition of calcium was not excessive.

As the deposition of calcium increased, there were several different sequences. In one instance, the calcium deposits seemed to shift from the interlamellar ground substance to the surfaces of regional structures (Figs. 3 and 4). The order of increasing affinity for calcium among these structures seemed to be as follows: first, elastic tissue; second, margins and processes of smooth muscle cells; third, collagen; fourth, fibrocytes and their processes. In another instance, interlamellar cells disappeared and calcium was deposited almost exclusively in elastic lamellas (Fig. 3). In the third instance, all structure was solidly incorporated in a diffuse deposit of calcium (Figs. 2, 4 and 8).

As stated previously, these sequences in calcification in the elastic arteries were found in two locations. The more common distribution was in the inner third of the media. Here, as the disorder progressed, there was essentially a wave of calcium deposition spreading deeply from the intima into the media (Figs. 3 and 6). The process resembled a slow diffusion from the blood toward the adventitia with decreasing amounts of calcium being deposited as the distance from the blood increased.

The second common distribution in the media was in the middle third of the wall. Here, there were discontinuous deposits which tended to encircle the vessel and to extend in the long axis, eventually merging with one another (Fig. 2). These were in the nature of enlarging bands of calcium deposits, which had a complex arrangement following the distribution of smooth muscle.

The best examples of elastic arteries with these lesions were the aorta, carotid arteries and pulmonary arteries. The changes were most conspicuous in the aorta. The inner wave of calcium deposition seldom spread into the outer third of the media of the proximal aorta (Fig. 3). With increasing distance from the aortic arch, the actual depth of penetration diminished slightly, but because of the decreasing thickness of the aortic wall, the relative depth of penetration increased. Hence, whenever calcium deposition extended beyond the inner third of the media of the aortic arch, the entire thickness of the wall of the lower abdominal aorta was usually calcified. The bandlike deposits of calcium were usually more prominent in the upper aorta. Though generally more common in the middle third of the media, the bands at times spread internally to the intima and externally to the adventitia (Fig. 4). In the latter instance, adventitial stromal reactions with vascularization of the media often occurred. Vascularization from the intimal side seldom occurred and was never conspicuous.

The pulmonary artery, except in the main trunk, was usually unaffected. Here, there were occasional focally distributed inner medial calcific plaques, rarely extending beyond the innermost 4 or 5 elastic lamellas. Extension of calcification into the secondary branches was encountered only in cases with severe alveolovenous calcific disease which in turn predisposed to chronic progressive emphysema.

The carotid arteries were usually severely affected. Though there was a recognizable combination of the inner and central patterns of medial disease, the inner pattern predominated and not infrequently spread, as in the lower aorta, throughout the thickness of the wall to the adventitia. We have no data on the changing pattern of the disorder along the course of the common carotid artery and its branches. However, the ophthalmic and cerebral arteries were always spared while the arteries to the salivary glands were usually severely affected.

Musculo-elastic Arteries. The pattern of lesions in the musculo-elastic arteries showed a gradient of change from the type found in elastic arteries to that encountered in large muscular arteries. In the axillary, common iliac, and upper femoral arteries, the inner and central patterns of medial alteration tended to merge with one another. Here, as the internal elastic membrane and smooth muscle became more conspicuous and the elastic tissue lamellas in the media less conspicuous, the pattern of medial alteration changed. The continuous wave of calcium deposition in the inner media was replaced by a periodically spaced local process which followed a spiral or bandlike distribution (Fig. 2). The earliest evidence of this process was either in or just beneath the internal elastic membrane (Figs. 6 and 7). It began locally as an interstitial swelling and fusion of fibrils. Calcium was deposited, and the deposits spread in depth as well as in the long axis of the vessel, with muscle cells often showing a remarkable persistence while the intercellular spaces and tissues were being occluded or impregnated with calcium. As the deposits spread, the contiguous calcified bands enlarged in depth and breadth, finally fusing to form a more or less continuous sheet of calcium from internal elastic membrane to adventitia (Fig. 8).

Muscular Arteries. The principal difference between different systems of musculo-elastic and muscular arteries was due to variation in the degree to which the media of the artery was affected by progression of the above process. For instance, the intensity of the process fell off very rapidly with successive divisions of the two primary coronary arterial branches, even though there was no significant change in vascular structure. On the contrary, the process increased in intensity along the course of arteries to the kidneys, the acid-secreting portion of stomach, the salivary glands, duodenum, colon and thyroid (Figs. 1 and 9). In other

systems it increased or decreased in an unpredictable manner in different animals. In still other systems, arteries were never affected, though they were of the muscular type with essentially the same structure or dimensions as arteries which elsewhere were regularly affected.

Arterioles and Capillaries. These vagaries of distribution were still more accentuated when attention was directed to the lesions in the media of arterioles. The distribution in arteriolar walls was essentially the same as in larger muscular arteries. As a rule, though, there was a greater degree of alteration from one arteriole to another in a given system than from one muscular artery to another in the same system. The earliest lesions, however, were in the same location, namely in a focal subintimal position. From here, the changes spread so that the walls of most affected arterioles became diffusely impregnated with calcium and were thicker than normal, with a corresponding reduction in the diameters of their lumens (Fig. 10).

The frequency and magnitude of involvement of arteriolar systems in different locations were in the following diminishing order: inner third of the renal cortex, acid-secreting part of the gastric wall, wall of the first part of the duodenum, salivary glands, spleen, skeletal muscle, cardiac muscle, renal pelvis, outer two thirds of the renal cortex, bone marrow, thymus, thyroid, colon, adipose tissue and pancreas. Arteriolar systems in the following locations were never involved: brain, eye, spinal cord, pituitary, adrenal, testis, liver, non-acid-secreting part of the gastric wall, esophagus, ileum, lung, ureter, fascia, tendons, synovial membranes, intervertebral disks and skin of the ear.

At times, there were lesions in the distal arterial system beyond the larger muscular arterioles. The changes, not necessarily associated with severe alteration of the larger arterioles, consisted of calcific deposits in the subendothelial tissues and membranes. Because of the thinness of the vascular walls, it was often impossible to recognize calcium deposits in capillaries beyond the terminal arteriolar system. Suffice it to say that such deposits were at times easily recognized in skeletal muscle, spleen, thyroid, acid-secreting gastric mucosa, bone marrow, thymus, and retroperitoneal fat tissue. It is possible that calcium deposits were usually there but were beyond the limits of detection by the microscopic methods used. The arteriolar-capillary deposits were especially interesting in the glomerular tufts (Figs. 9 and 10). Here, isolated arteriolar loops were primarily affected. Occasionally, most of the glomerular arteriolar structure was involved so that subendothelial membranes were sharply outlined by calcium deposits, but glomerular alterations occurred only when the arterial system leading to the glomerulus was also calcified. In the spleen the calcium deposits occasionally extended

beyond the limits of arterioles and impregnated the reticular structure of lymphoid follicles while the large vascular sinuses of the red pulp were spared. In the thyroid, skeletal muscle and other previously specified locations, the walls of capillaries were occasionally outlined by thin subendothelial lines of calcium, but this was usually recognizable only in animals with severe generalized disease, especially in small arteries and precapillary arterioles in tissues showing capillary calcification.

Internal Elastic Membrane

There were two divergent alterations in the internal elastic membranes of musculo-elastic and muscular arteries or arterioles. The usual type of change was characterized by the following sequence. First, the customary regular undulation of the membrane was replaced by an irregular undulation with linearity along short stretches (Figs. 5 and 6). These zones, which were usually discontinuous but often regularly spaced in the circumferential and long axes of the vessel, acquired an affinity for hematoxylin, interpreted as evidence of early calcification (Figs. 6 and 7). This was followed by a tendency for transverse discontinuities or fractures to develop, and calcium deposits became increasingly conspicuous in or on the membrane (Figs. 5 to 7).

The second type of lesion in the membrane resembled the one just described up to the point of modification of the staining affinities. Then, rather than acquiring an increasing affinity for hematoxylin, the membrane developed an increased affinity for eosin (Fig. 5). At the same time, its optical properties changed and it became more homogeneous and refractile. This modification was not accompanied by development of sharp transverse fractures. On the contrary, the discontinuities which appeared as this process increased in severity were the result of attenuation and fraying of the membrane (Fig. 5).

A detailed analysis of the distribution of the two types of lesion in the internal elastic membrane has not been made. Some arterial and arteriolar systems were more susceptible to one type of change than the other. It was expected that the nature of the alteration in the internal elastic membrane might reflect the nature of changes in the elastic membranes in the media. This was not found. The lesions in the media were essentially the same, irrespective of the direction of change in the internal elastic membrane. There was, however, a fairly consistent spatial relation between the deteriorative alterations in the membrane and degenerative-calcific changes in the subjacent medial structure (Figs. 5 to 7). This was easily recognized in the femoral, brachial, splenic, gastric and other large muscular arteries.

Intima

Pathologic changes in the intima were spatially related to alterations in the internal elastic membrane and subjacent media. The sequences in the development of these lesions were as follows: The first recognizable changes were in the internal elastic membrane and subjacent media as previously described. (Fig. 5). These were usually focal and spaced rather regularly. As they increased in severity, proliferative reactions often occurred in the intima. The early proliferative reactions were essentially fibroblastic in character (Fig. 5). The final product of the reaction was usually a diffuse or focal avascular fibrous structure which at times was as thick as the original vascular wall (Figs. 6 to 8). In some instances, newly formed elastic tissue appeared among the collagenous elements. At other times, there was a differentiation of the proliferating fibroblasts into smooth muscle cells. This was common in the large renal, iliac and femoral arteries. At still other times, the thick avascular intima showed degeneration in cells and stroma, with accumulation of lipids and calcium. As a rule, however, the proliferative reactions were essentially in the direction of the formation of fibrous plaques of increasing thickness, apparently limited by a surprisingly consistent refractoriness to vascularization (Fig. 8).

A curious feature of this reaction was the lack of correlation between the degree of intimal proliferation and the degree of abnormality of the internal elastic membrane and media. In other words, though the intimal proliferation was always spatially related and temporally secondary to the subjacent disorder, the occurrence of severe changes in the internal elastic membrane and media was often unaccompanied by any intimal proliferative reaction. Several factors seemed to govern this matter. First, some vessels were more reactive than others. For instance, intimal proliferation in the arch of the aorta was much less than the proliferative reaction in the low thoracic and abdominal segments (Fig. 3). Also, proliferative reactions were conspicuous in large muscular branches such as the iliac, renal, femoral, splenic and gastric arteries (Figs. 2 and 6 to 8). The reactions were usually minimal or absent in small muscular arteries and arterioles even in the presence of severe changes in the internal elastic membrane and the media (Figs. 9 and 10). Second, the distribution and rate of development of degenerative-calcific alterations in the vascular wall seemed to influence the intimal reactions. Local changes were a greater stimulus than general changes. Slowly progressive lesions were a greater stimulus than acute transient lesions. Finally, the development of sharp discontinuities in the internal elastic membrane, especially of muscular arteries, was an important stimulus

to intimal proliferation (Figs. 5 to 7). In these instances the fractures in the membrane widened, and the defects so created were filled in by fibroblasts and collagen which spread progressively in an apparent attempt to bridge the defects (Fig. 5). This led to the formation of separate or fused continuous intimal plaques which required several weeks to reach maximum thickness. In addition to the factors mentioned, intimal proliferation was regulated by other conditions which are still obscure. One of these was the alternation of periods of high viosterol dosage with periods during which viosterol was not given. A chronic intermittent state of hypervitaminosis D was a more effective stimulus to intimal proliferation in small arteries than a chronic persistent state. In no case, however, did the newly formed intima exceed the thickness of the arterial wall (Figs. 6 to 8).

One other form of intimal reaction should be mentioned. It consisted of enlargement of single isolated endothelial cells. These cells had a strong affinity for hematoxylin, and as this affinity increased, each cell was transformed into a series of concentric sphero-crystals with the nucleus of the cell at the center and the cytoplasmic membrane at the periphery. These cells were conspicuous in renal glomeruli and often were the only part of the glomerulus involved by calcium deposits. They were found at times in other small vascular channels, especially in the lungs. Here, they were most easily recognized in the mucosal vessels of the trachea and large bronchi. In this location they were usually in the endothelium of the vascular wall adjacent to stretches of calcium deposits in the subepithelial tissues of the respiratory mucosa. The significance of these cells was not clear. Their location and structure indicated that they belonged to the reticuloendothelial system. Presumably, they had been activated to engulf calcium and had progressively accumulated calcium in sufficient quantities to undergo crystallization as the cell became nonviable. There was no clue as to why the reticuloendothelial system should have these activities restricted to only a few special locations.

DISTRIBUTION OF THE DISORDER IN DIFFERENT ARTERIAL SYSTEMS

The pathologic alterations appeared first in the proximal aorta. With increasing duration and intensity of the disorder, the changes spread along the aorta and became more conspicuous in its principal branches.

The same general pattern of spread was encountered in the coronary arterial system. Lesions appeared first near the arterial orifices, and here medial disease was often very pronounced. The magnitude of the process decreased in the direction of the blood flow in most instances, but in some cases with active or healed arteritis and myocardial calcification, the intramyocardial branches were more severely affected than the larger vessels.

The common carotid arteries were regularly involved whenever there was a significant amount of aortic abnormality. Pathologic changes usually decreased in the direction of the blood flow. Somewhere along the course of the internal carotid artery, lesions disappeared, because no abnormality was found in the ophthalmic or cerebral arteries. The disorder persisted to a variable degree along the course of the external carotid artery and its branches. This persistence was selective because alterations in the arteries of the submaxillary gland were far more severe than those encountered in other branches of the external carotid arterial system.

The branches of the thyroid axis were not examined in detail, but the thyroid artery was always involved in moderately severe cases. The vertebral arteries were not examined distally, but in no case was there any abnormality of the basilar artery.

Lesions in the axillary, brachial, iliac and femoral arteries were similar. They decreased in severity in the direction of the blood flow. The magnitude of disease at comparable levels was greater in the arteries of the lower than upper extremities. As a rule, evidence of the disorder decreased to zero along the first or second divisions of the muscular branches of the brachial and femoral arteries. However, in most instances of arteritis or calcific myositis, alterations in arteries again became conspicuous as the extramuscular branches passed into the muscle. Successively smaller intramuscular divisions then showed lesions of increasing severity.

The large branches of the descending aorta were usually involved although the intercostal arteries seldom had significant changes. The branches of the celiac axis were about equally affected at their origins. The severity of the process increased along the course of the left gastric and splenic arteries while it decreased along the course of the hepatic artery. Divisions of the hepatic artery entering the liver and all subsequent branches were normal. Divisions of the splenic artery leading to the pancreas showed diminishing alteration and a transition to normal vessels at the lobular level. Divisions of the splenic artery entering the spleen frequently showed increasing severity of lesions down into the terminal arterioles in lymphoid follicles. Divisions of the left gastric artery showed a persistence of moderate abnormality which increased greatly in severity along the branches which penetrated the muscular wall of the acid-secreting part of the stomach. With subsequent divisions down to arterioles passing just beyond the muscularis mucosae into the mucosa, the disorder increased progressively in severity but again diminished in the smaller mucosal vessels between the glands. The distribution of these vascular changes was precisely defined. They did not

extend beyond the acid-secreting mucosa or the subjacent submucosa, muscularis mucosae, and tunica muscularis. All arteries in the remainder of the stomach, especially of the pyloric region, were normal. However, just distal to the pylorus, the mucosa and subjacent wall of the first part of the duodenum were involved by arterial lesions similar to and only a little less severe than those in the acid-secreting part of the gastric wall.

The superior and inferior mesenteric arteries were not regularly examined microscopically, but there were often gross alterations which diminished along the course of the branches. A study of the smaller divisions in sections of the ileum and colon showed minimal changes or normal structure. No arteriolar involvement was recognized in the ileum, but in the colon severe lesions were occasionally conspicuous.

The main adrenal arteries were not examined regularly, but there was no arterial abnormality in the adrenal glands.

The main renal arteries were less severely affected than other major branches of the aorta. The principal divisions usually showed a slight to moderate alteration, but as these branches divided to enter the kidney near the corticomedullary junction (Fig. 1), severe arterial lesions became conspicuous and spread along the first intracortical branches and arterioles leading to the proximal glomeruli (Figs. 9 and 10). As the nephrons adjacent to the corticomedullary junction became inactivated, the disorder spread in continuity along the main trunks, branches and preglomerular arterioles toward the peripheral third of the cortex but seldom affected it except in instances of prolonged chronic disease.

Arterial systems of the testes, ureters, skin, bone, skeletal muscle, brain, spinal cord, salivary glands and other structures were regularly investigated. There was no abnormality of arteries in or at the hilus of the testis. The arteries and arterioles along the ureters were normal. The arterioles and precapillary arterioles in the skin of the abdomen were occasionally involved but only in the most severe cases. Only the large central artery of the ear ever showed abnormality. The arteries and arterioles in the bone marrow were regularly affected while those to synovial membranes and intervertebral disks were spared. Changes in the vessels in skeletal muscle were usually found in many locations. The brain, spinal cord and their membranes had no trace of vascular disease. The large arteries leading to the salivary glands were often either normal or only slightly involved by the process. As these divided into multiple branches within the gland, the severity of the disorder often increased distally so that many small arteries and arterioles along the minor ducts were solidly calcified, and the lesions could occasionally be traced into the walls of the smallest vascular divisions between the

secreting glands. Under these conditions atrophic changes in the lobules of the gland became conspicuous, and, at times, basement membranes of atrophic glands were outlined by deposits of calcium.

The general distribution of lesions in the pulmonary vascular system also deserves some comment. The main trunk of the pulmonary artery occasionally had a few scattered superficial foci of calcification in the inner media. These were less common in the two main branches. Except when there was chronic pulmonary emphysema, the intrapulmonic branches remained normal to the level of the interalveolar capillaries. Then, in some animals, calcium deposits appeared in the walls of the capillaries. These deposits were irregularly distributed and were conspicuous only in animals with severe generalized disease. The capillaries were tributaries to small venules and thence to veins which also had a variable but often severe degree of degenerative-calcific medial alteration. Ordinarily, these lesions continued along the main pulmonic veins to the left auricle of the heart. The venous changes were reactionless and, in contrast to arterial lesions, were unaccompanied by intimal proliferative responses.

Discussion

Generalized arteriosclerosis in man is represented by many combinations of different types and distributions of chronic vascular lesions. As a rule, the lesions are noninflammatory in nature and they exhibit a mixture of chronic progressive degenerative and reparative sequences which become more conspicuous with advancing age.[10–12] The disorder is never identical in any two people. This lack of uniformity has stimulated experimental work designed to segregate and analyze its elementary aspects. One aspect is intimal atheromatosis, and this has been investigated in several animal species with hyperlipemia or hypercholesteremia induced by different methods.[2] A second aspect is intimal fibro-elastosis; this has been studied principally by creating conditions which cause local inflammation or degeneration of the arterial wall. Still another aspect is medial degeneration. This has been produced locally and systemically by many methods.[1,4,12,13] As a rule, however, published descriptions of the distribution and evolution of experimental vascular lesions have not been given in sufficient detail to justify comparisons with analogous lesions in the common forms of human arteriosclerosis. We are confronted, therefore, with many vascular alterations which can be produced experimentally but with little evidence that these have any relation to generalized arteriosclerosis in man. The deficiency of evidence is not only in absence of proof of a common pathogenesis but also in lack of proof of a common pattern of structural change.

The occurrence of arteriosclerosis in animals or man with hypervitaminosis D does not justify an assumption that this disorder is connected with the pathogenesis of the usual forms of human arteriosclerosis. The occurrence may justify an assumption, however, that a metabolic derangement similar to that in hypervitaminosis D but of another origin may be a factor in the pathogenesis of human arteriosclerosis. This assumption may be strengthened by emphasizing the similarity and minimizing the dissimilarity of pathologic changes in the experimental and human disorders.

The first basic point of similarity is the tendency for both disorders to be generalized throughout the major divisions of the systemic arterial system.[7] This tendency was less pronounced in the experimental disorder than is the rule in the common forms of human arteriosclerosis. It must be remembered, however, that the duration of the experimental condition was brief, and had it been extended throughout the normal life span of the animal, a more widespread distribution might have occurred. At least, long duration seems to be an important factor in the evolution both of human arteriosclerosis and experimental hyperlipemic atherosclerosis.[8]

A second point of similarity is the tendency for lesions in this experimental disease and human arteriosclerosis to be of maximal severity in certain arterial systems and minimal or absent in other arterial systems having essentially the same dimensions and structure. In man, the relative magnitude of the disease in different systems follows no consistent pattern from patient to patient.[9-11] In the experimental disorder the relative magnitude of disease in different systems was surprisingly uniform in animals subjected to the same regime. Even though the relative magnitudes of involvement of different arterial systems in man varies from person to person, there are certain systems which are statistically more susceptible to arteriosclerosis than others. In general, the aortic, coronary, renal, cerebral, femorotibial and splenic systems are among the more susceptible ones.[9] In the experimental animal, all these except the cerebral and tibial arteries were also quite susceptible.[6] In man, the intrahepatic, pulmonic intra-adrenal, intramyocardial, dermal, hypophysial and bone marrow systems are among the least susceptible.[9] In the experimental disorder this was also true with the possible exception of the intramyocardial and bone-marrow systems.[6]

Although these comparisons indicate some differences in the elective distribution of the experimental and the human lesions, they fail to emphasize that, exclusive of arteritis, the major pathologic changes in the peripheral arterial systems of animals were in the kidney, the wall of the acid-secreting part of the stomach, the wall of the duodenum,

the spleen, the salivary glands and the pulmonary venous system. Such a distribution of major peripheral vascular changes never occurs in man, except perhaps in rare instances of hyperparathyroidism, hypervitaminosis D, or other conditions productive of the syndrome known as "calcium metastasis." A careful examination of this syndrome, however, discloses that the magnitude and distribution of lesions cannot be accounted for by the theory intended to explain them.[14] Furthermore, by appropriate control of the degree and duration of hypervitaminosis D, a pure vascular disorder without resemblance to "calcium metastasis" in its distribution can be produced.

The third point of similarity between the experimental and human disorders is the tendency for the intensity of the vascular lesions to vary greatly among similar branches of an affected arterial system. This variation is always present in vessels of all dimensions in human arteriosclerosis where two arterioles of identical structure arising from a single vessel are commonly seen. One arteriole may show severe alterations along its course, and the other may retain a normal structure. This characteristic of human arteriosclerosis was equally conspicuous in the experimental disease. It was well shown in small arteries and arterioles in cardiac muscle, skeletal muscle, renal cortex, salivary glands and gastric wall. It was especially impressive in the arborization of radial arteries in the renal cortex. Here, in the same microscopic field, normal and severely affected arteries, arterioles, and glomerular tufts were encountered side by side, the overall degree of involvement diminishing as the periphery of the cortex was approached.

A fourth point of similarity between the two disorders is that the degenerative sequences and processes of repair are occasionally complicated by inflammatory reactions. In human arteriosclerosis these reactions are seldom conspicuous in the smaller divisions unless some unusual factor is superimposed. In the larger divisions, inflammatory reactions are common, especially in the adventitia of the aorta and coronary arteries, when the mural degeneration is severe and accompanied by more than the usual amount of necrosis or mural thrombosis with vascularizing reaction. Secondary inflammatory reactions were also occasionally encountered in the experimental disorder, but the common primary reactions which either accompanied or preceded the degenerative-calcific changes in arteries in the myocardium and in skeletal muscle are not a feature of human arteriosclerosis. These intramuscular arteritic and periarteritic reactions were not a necessary feature of the experimental disease, for they were dependent on an excessive dosage regime and perhaps an intercurrent pulmonary infection. Hence, there was no evidence that inflammation in the usual

sense was any more significant in the pathogenesis and evolution of the experimental disorder than in human arteriosclerosis.

The final important point of similarity is the close resemblance between the microscopic sequences in the Mönckeberg type of human arteriosclerosis and sequences in the evolution of the experimental disease in larger muscular and musculo-elastic arteries.[15] The Mönckeberg type of deterioration and calcification of the internal elastic membrane and media with subsequent intimal proliferative reactions was reproduced in close detail in the experimental animals. There was, however, one conspicuous difference between the degenerative-calcific condition of the media of human arteries and that of the experimental animals. In man, the degenerative-calcific changes tend to gradually decrease as the arterial branches of an affected system decrease in dimensions. Calcium deposits become hardly recognizable in small branches, and the degenerated walls of small arteries and arterioles often acquire a homogeneous hyaline property characterized by an affinity for eosin and an accumulation of lipids. In the experimental disorder there was a tendency for the degenerative-calcific lesions to spread beyond the large divisions and involve the small arteries and arterioles. Although the walls of these small vessels underwent structural alterations similar to those commonly encountered in human arterio-arteriolosclerosis, they acquired an affinity for hematoxylin, rather than for eosin, as is characteristic in human disease. At times, however, the affinity for hematoxylin was changed to an affinity of the degenerated structure for eosin. This change was always accompanied by regional resorption of calcium deposits. At other times, the internal elastic membranes of certain arteries seemed to be transformed directly into eosinophilic hyaline structures which differed sharply from the subjacent medial elastica, deeply stained with hematoxylin. Despite these suggestive trends, the degenerated eosinophilic hyaline medial structure, characteristic of human arteriolosclerosis, was never found in the experimental disorder.

It would seem from the foregoing comments that hypervitaminosis D, as well as pyridoxine deficiency, may be useful as a tool for investigating the pathogenesis of some elementary arterial structural lesions similar to those occurring in human arteriosclerosis.[12,13] Among these, the degeneration and calcification occurring in the media of arteries are conspicuous. It has been assumed that these changes in animals are a direct result of an increase in serum calcium and a "toxic" action of Vitamin D.[6–8] If so, all vessels are not equally affected. Furthermore, animals used in these experiments had no persistent or consistent rise in the average levels of serum calcium, though there was ordinarily an upward trend in the serum inorganic phosphorus. The magnitude of calcific

medial disease was not related to alterations in the levels of serum calcium and inorganic phosphorus. This points out the desirability of seeking for other factors responsible for development of calcific degenerative arteriosclerosis in hypervitaminosis D. It is possible that these factors, when fully disclosed, may also bear upon the pathogenesis of senile calcific degenerative arteriosclerosis in man.[16]

This abnormal metabolic state also offers an opportunity to analyze another aspect of arteriosclerosis, namely the variable intimal proliferative reactions. In the experimental animal these followed degenerative-calcific changes in the internal elastic membrane and subjacent media. These alterations, however, were not the sole determinants of the rate or amount of fibro-elastic intimal proliferation.[12] This is also true in human arteriosclerosis. In other words, there is no parallelism between the magnitude of calcific medial disease and the degree of intimal proliferation secondary to medial degeneration in man or animal. Though these differences may be due to a variation in the deposition of fibrin on the intima, the deposits, if present, were not recognized by the methods used.[17,18]

Finally, this experimental disorder provides a model system for a study of relations between medial calcific degeneration, intimal fibro-elastic proliferation, atheromatous deposition and thrombosis. One approach might properly include a study of the effects of combinations of hypercholesteremia, hyperlipemia and hypervitaminosis D.[19] A second approach might be concerned with chronic states involving prolonged repair of the effects of these combinations, for it seems probable that the occurrence of occlusive arterial thrombosis in human atherosclerosis is usually secondary to disturbance of vascularizing stromal repair of chronic degenerated intimal lesions.[20,21] Hence, by prolonging the experiments, it might be possible to induce a state of intimal vascularization and thrombosis, neither of which has occurred in these experiments despite very severe intimal arterial disease with extensive medial vascularization (Fig. 8). A third approach deals with a larger problem. This is concerned with a study of mechanisms by which the pattern of distribution of organic vascular disease may be governed by the pattern of distribution of units of tissue structure whose coordination is required for the performance of function.[9] This is clearly evident in hypervitaminosis D and there are reasons for believing that the variations of organic vascular disease in different arterial systems among different people may have a similar pathogenesis. In any event, a means is hereby provided for assessing the role of function and regulation of function in the etiology of organic disease of arterial sys-

tems which supply tissues whose coordinated activity is required for carrying out the function.

Summary

Rabbits given excessive doses of irradiated ergosterol developed a generalized disorder characterized principally by resorption of bone and abnormal deposition of calcium salts in many extra-osseous tissues. The circulatory system was particularly susceptible to calcification, but there was a wide variation in lesions in different parts of the system. On a moderate dosage regime, calcium appeared first in the inner media of the aortic arch. With increasing time the deposits spread in depth and in the direction of the blood flow along the aorta and into the major branches. Usually, arterial changes decreased in severity in successively smaller branches, but there were two principal exceptions to this rule. The first exception occurred in animals on high dosage regimes. These animals developed a conspicuous arteritis and periarteritis in cardiac and skeletal mucle. These inflammatory reactions either accompanied or served to augment the development of intramuscular degenerative-calcific disease of arteries and arterioles. The second exception occurred in special arterial systems where the calcific vascular disorder increased in severity in the successively smaller divisions. This was conspicuous in the spleen, the inner third of the renal cortex, the duodenum, the salivary glands, and the part of the gastric wall concerned with acid secretion. In the pulmonary circuit the arterial system was usually unaffected while the pulmonary venous system from the interalveolar capillaries to the left auricle was often affected by the disease.

The basic histologic alterations formed a complex of inflammatory-degenerative-calcific sequences with subsequent reparative reactions. The principal locus of the deteriorative-calcific lesions was in the internal elastic membrane and media while the fibro-elastic intimal proliferative reactions and vascularized stromal resorption of the media were the principal manifestations of repair. Combinations of these changes led to the development of structural forms of vascular disease essentially identical to those encountered in the Mönckeberg type of human arteriosclerosis. This similarity, together with the remarkable distribution of the disorder, characterized by conspicuous susceptibility of certain arterial systems and absolute resistance of others, indicated that the disease should be useful in the study of factors which may contribute to the pathogenesis of human arteriosclerosis. Not the least important among these factors would seem to be the mechanisms which regulate the blood supply to functionally coordinated units of structure.

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We wish to acknowledge the preparation of the microphotographs by Mr. Jack deBruin.

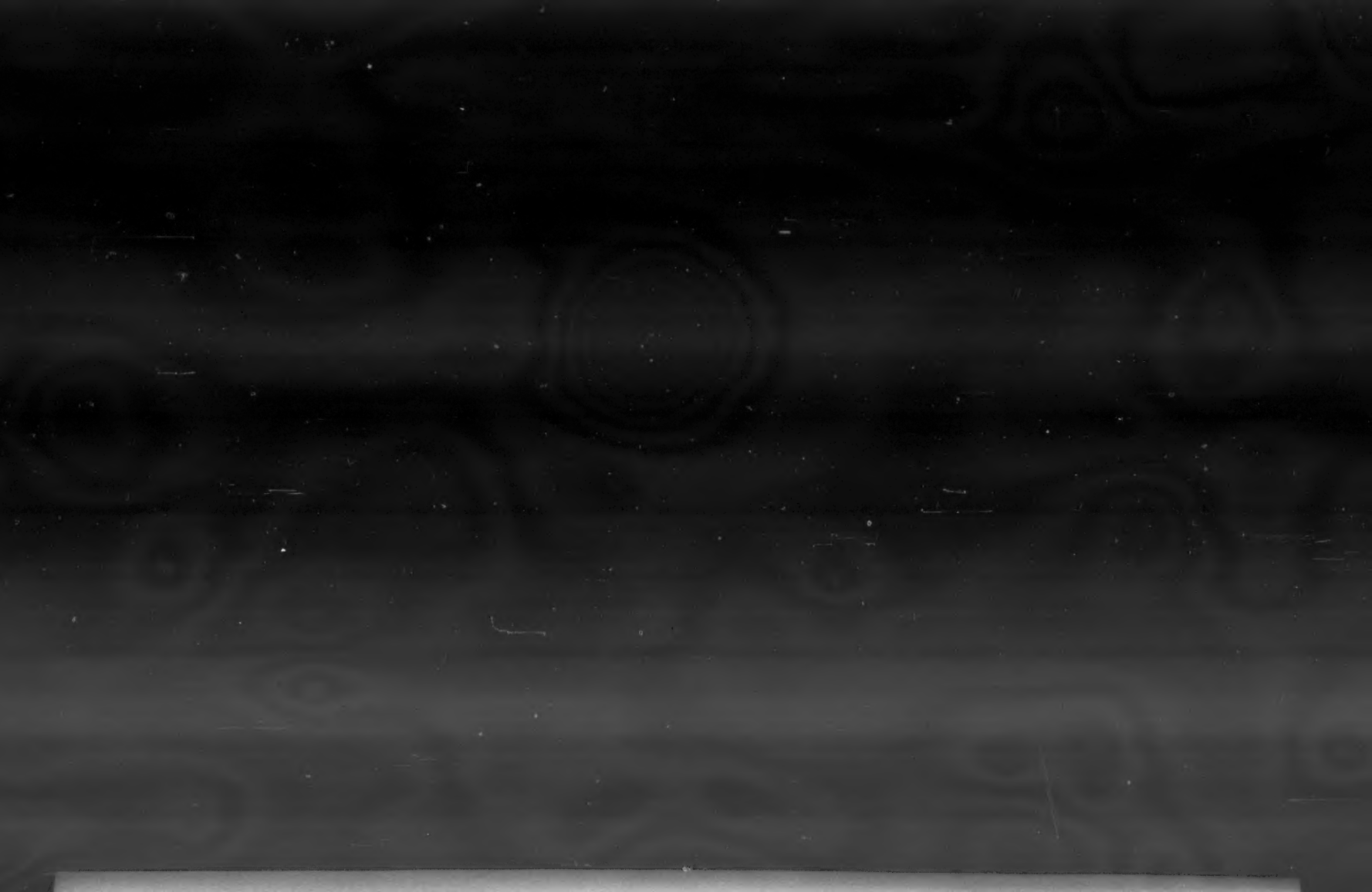
[*Illustrations follow*]

Legends for Figures

Photographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. Several extraparenchymal branches of a renal artery in a rabbit given 200,000 units of viosterol for 6 weeks and sacrificed at the end of 6 additional weeks on a normal regime. There is extensive calcification of the internal elastic membranes and subjacent media as indicated by the darkly stained parts of each vascular wall. An associated secondary intimal proliferation can be detected in several places. × 20.

Fig. 2. Iliac artery in a rabbit given 200,000 units of viosterol each week for 6 weeks. The deeply stained areas in the media are discontinuous concentric rings of calcium deposit. Over each of these and restricted to them is a plaque of thickened fibrous intima, in places equal in thickness to the thickness of the normal arterial wall. × 40.



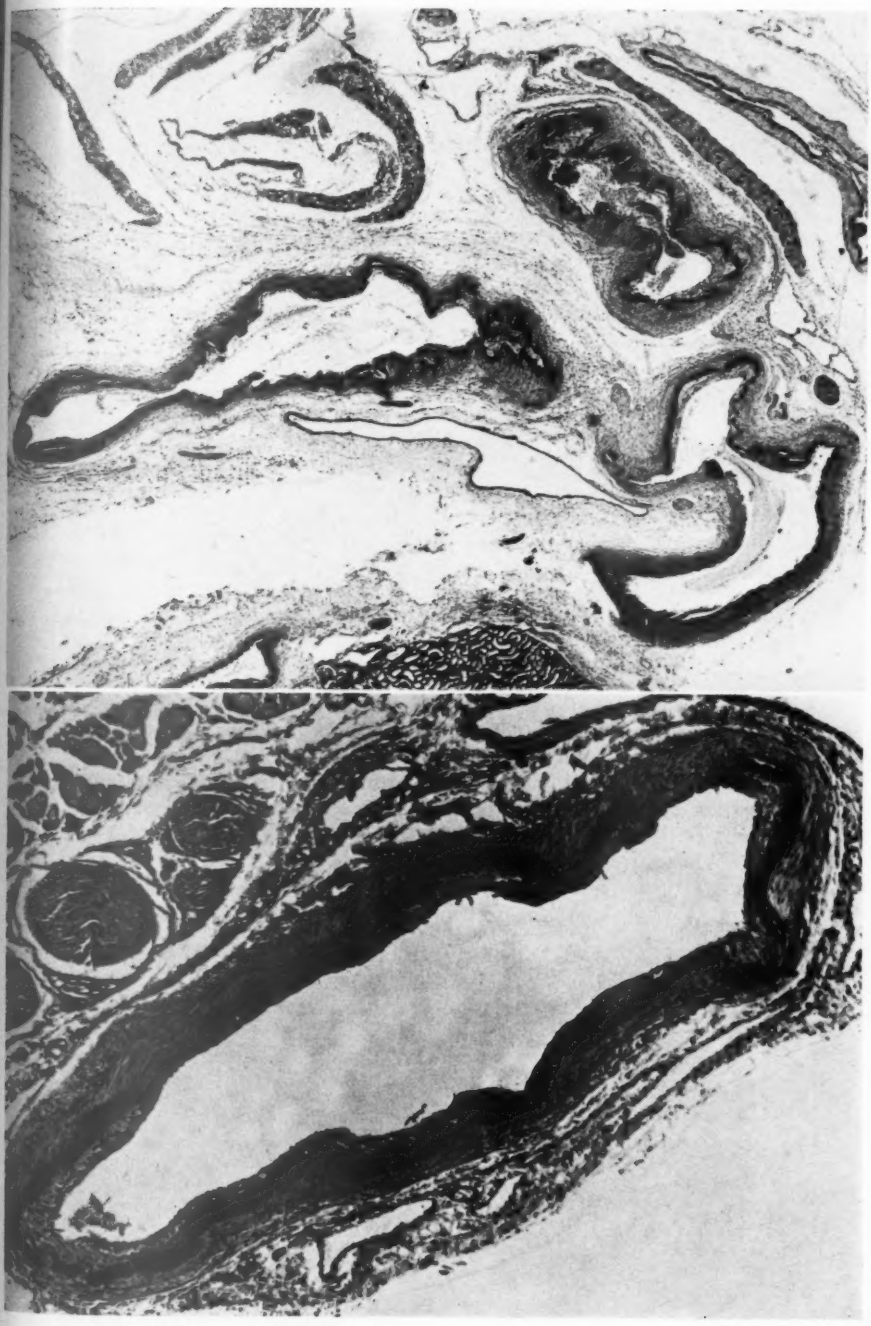


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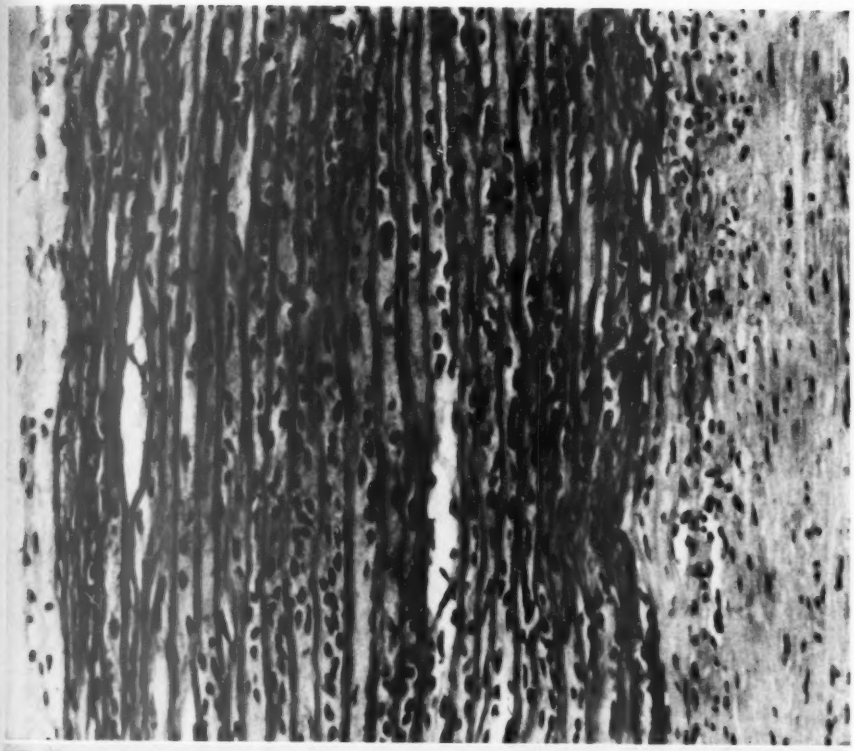
FIG. 3. Full thickness of the wall of the thoracic aorta in a rabbit given 200,000 units of viosterol every fourth week over a period of 33 weeks. The inner two thirds of the media shows calcium deposits restricted largely to the elastic lamellas. There is minimal intimal proliferation. Structural relations and cellular elements are well preserved throughout the media. × 180.

FIG. 4. Full thickness of the media and intima of the abdominal aorta in a rabbit given 200,000 units of viosterol every fourth week for 42 weeks. An area of medial calcification similar to that shown in Figures 2 and 8 has been penetrated by vascularized mesenchyme, arising in the adventitia and extending through the media to the thickened intima. Resorption of calcium and degenerated medial structure is advanced. These medial vascularizing sequences seldom, if ever, originated from the intimal aspect of arterial lumen. × 420.



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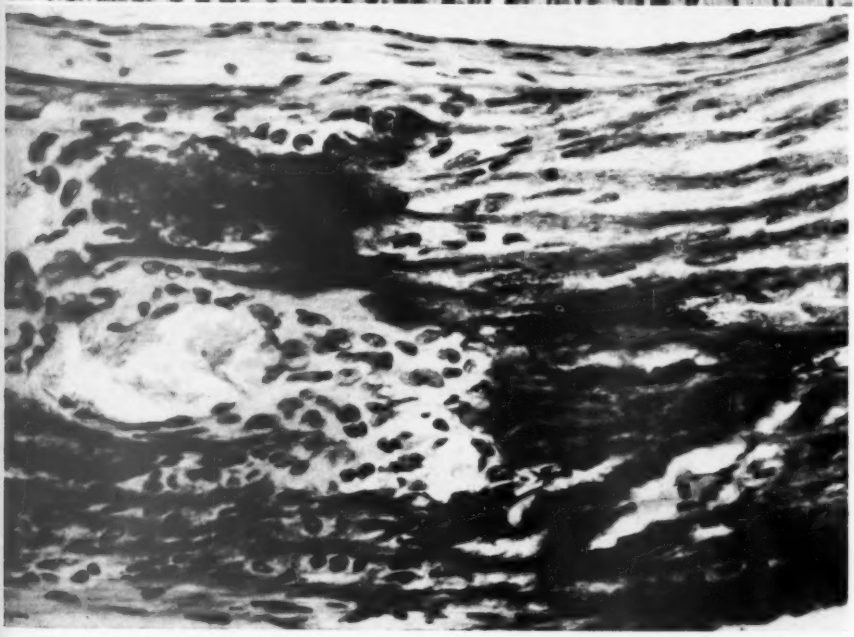

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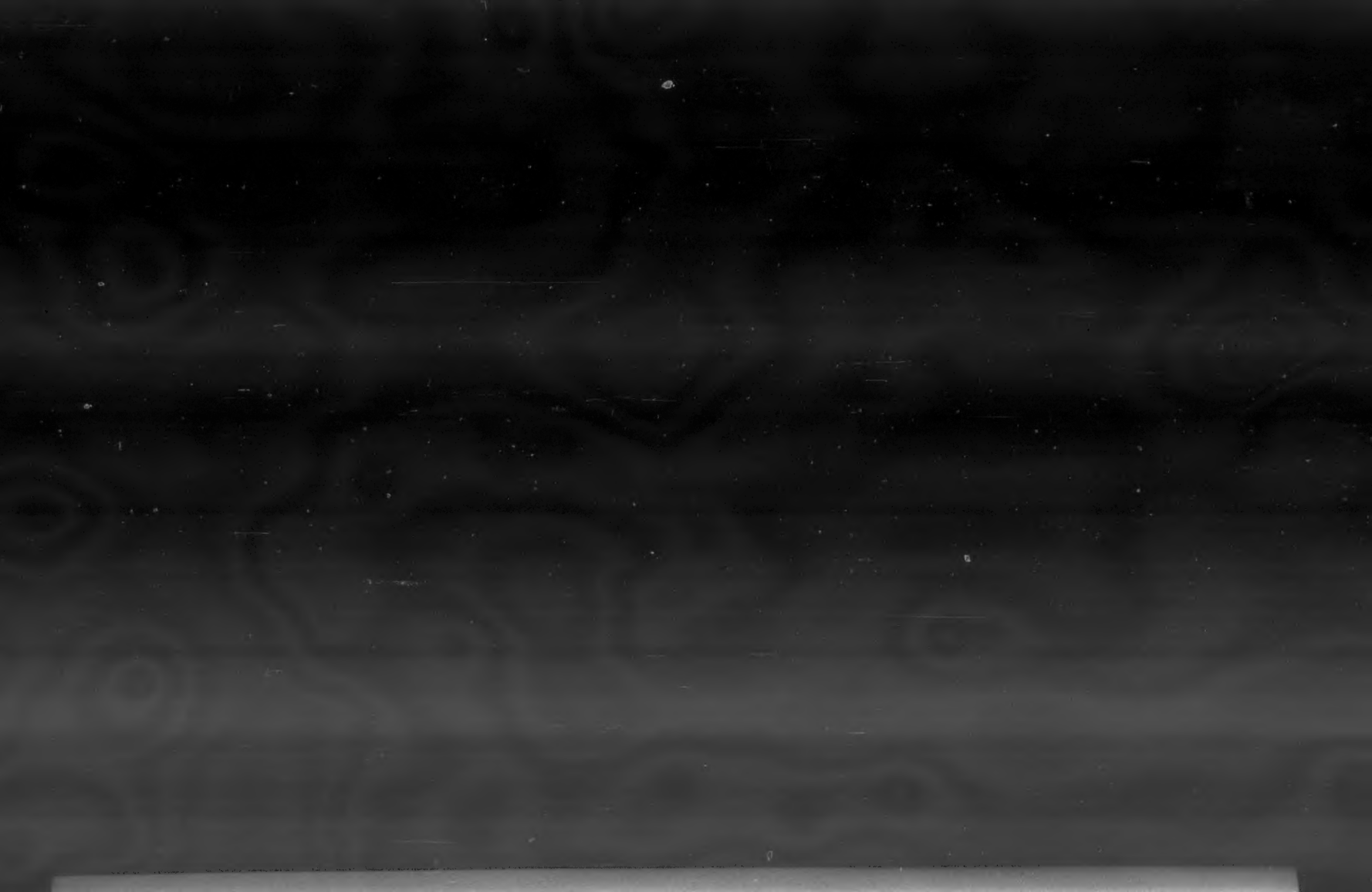
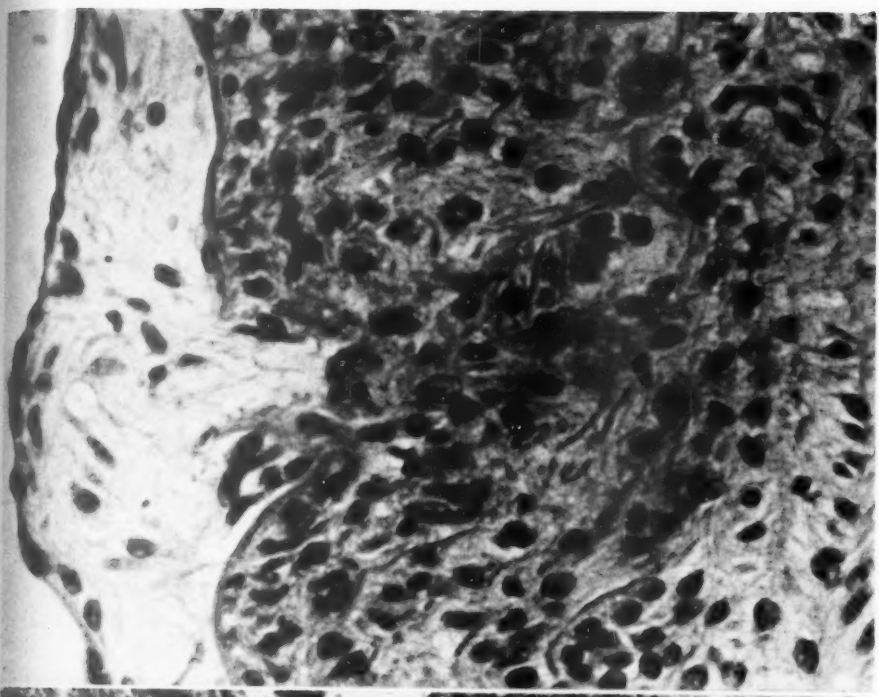
FIG. 5. Carotid artery in a rabbit given 200,000 units of viosterol each week for 4 weeks. There is focal subintimal medial degeneration with early calcification and mesenchymal activation. The internal elastic membrane is hyalinized and has an affinity for eosin. It is fractured and frayed over the site of medial degeneration. At this point there is a "splint" of proliferating intima covered with a regenerated endothelium. The fibroblasts in the immature plaque are separated by a myxomatous nonfibrillar matrix. It is possible that a small thin layer of fibrin may have provided the initial lattice for support of the migrating fibroblasts. If so, it must have been promptly resorbed. This represents one of the earliest stages of primary medial degeneration and secondary intimal proliferation. × 48.

FIG. 6. Femoral artery in a rabbit given 200,000 units of viosterol each week for 4 weeks. All but a short segment of the internal elastic membrane and subjacent media has undergone degeneration and early calcification. Though there is some cellular reaction in the media, the principal response is an intimal mesenchymal activation with fibrocellular intimal thickening, almost equal to the thickness of the media. At the angle, the media, internal elastic membrane and intima retain normal structure. × 150.





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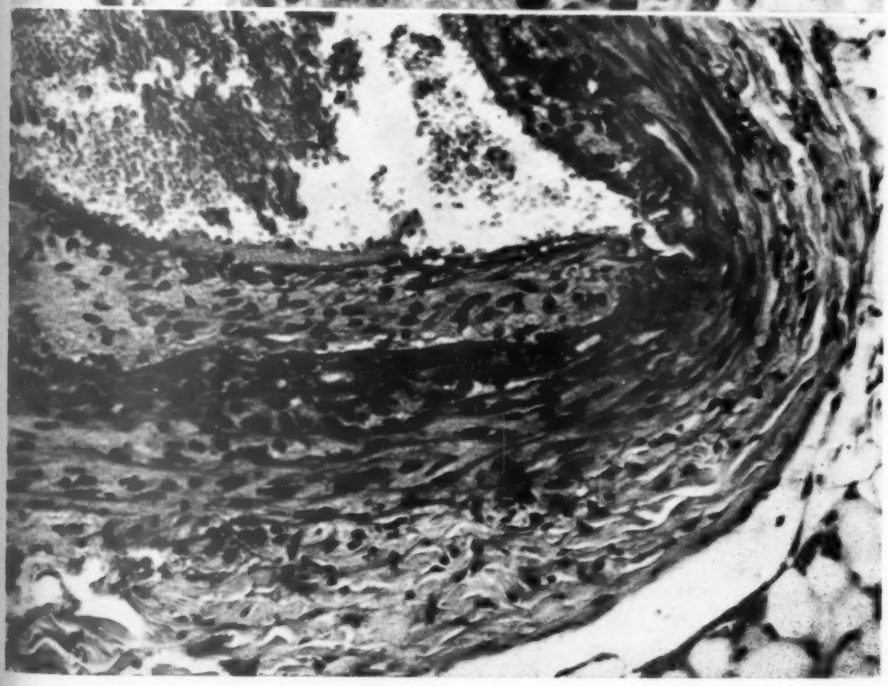

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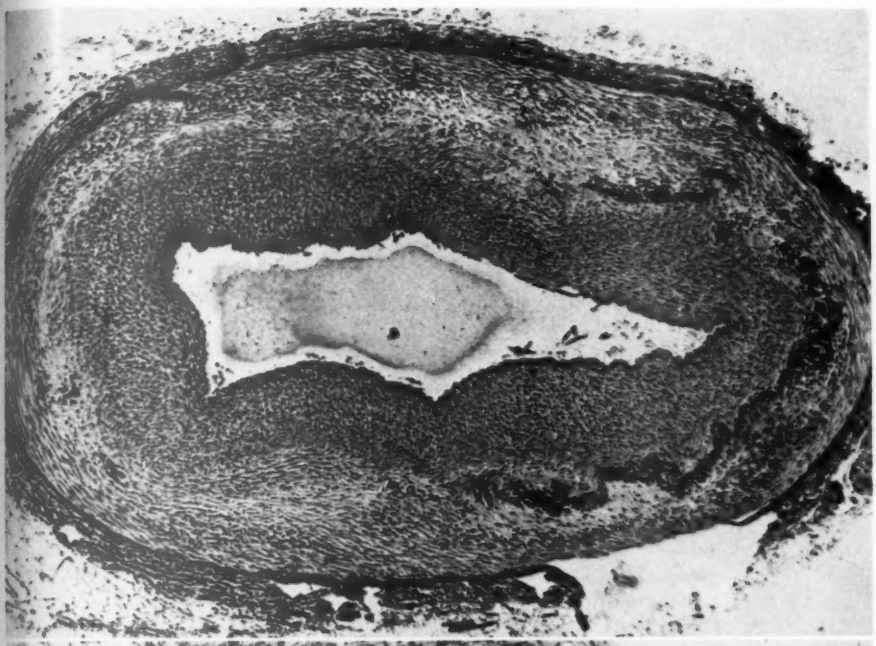
FIG. 7. A main renal artery in a rabbit given 200,000 units of viosterol every fourth week for 54 weeks. The remnants of the irregular, discontinuous, darkly stained, calcified internal elastic membrane now lie in the middle of the arterial wall. Internal to this is a greatly thickened fibrocellular intima, which has encroached upon the lumen. External to the fragmented membrane is a partly degenerated scarred media with remnants of calcium deposits undergoing avascular resorption. The external elastic membrane, though calcified, as shown by its affinity for hematoxylin, is intact and has acted as a barrier to adventitial mesenchymal penetration of the media. As usual, the thick, fibrocellular, newly formed intima is refractory to calcification despite prolonged viosterol dosage. These changes closely resemble those often found in medium-sized muscular arteries in the human being. × 50.

FIG. 8. Cross-section of a femoral artery in a rabbit given 200.000 units of viosterol every third week over a period of 33 weeks. The lumen is partly filled with a clot. It is lined by regenerated endothelium supported by thick dense fibrocellular intima, resistant to calcification. External to the thick intima is a porous, mottled zone once occupied by a medial smooth muscle and now by densely calcified, darkly stained degenerated medial structure being resorbed by highly vascular mesenchyme which has penetrated the media from the adventitia. Despite the intense vascularization and associated resorption of the degenerated calcified media, not a single capillary could be found in the thick fibrous intima. × 40.



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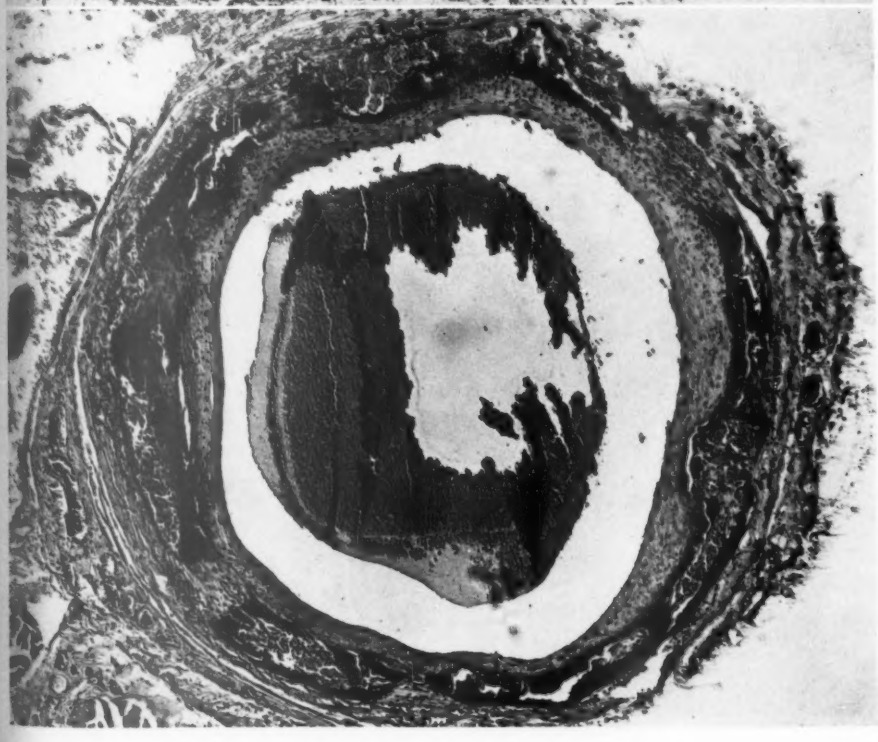

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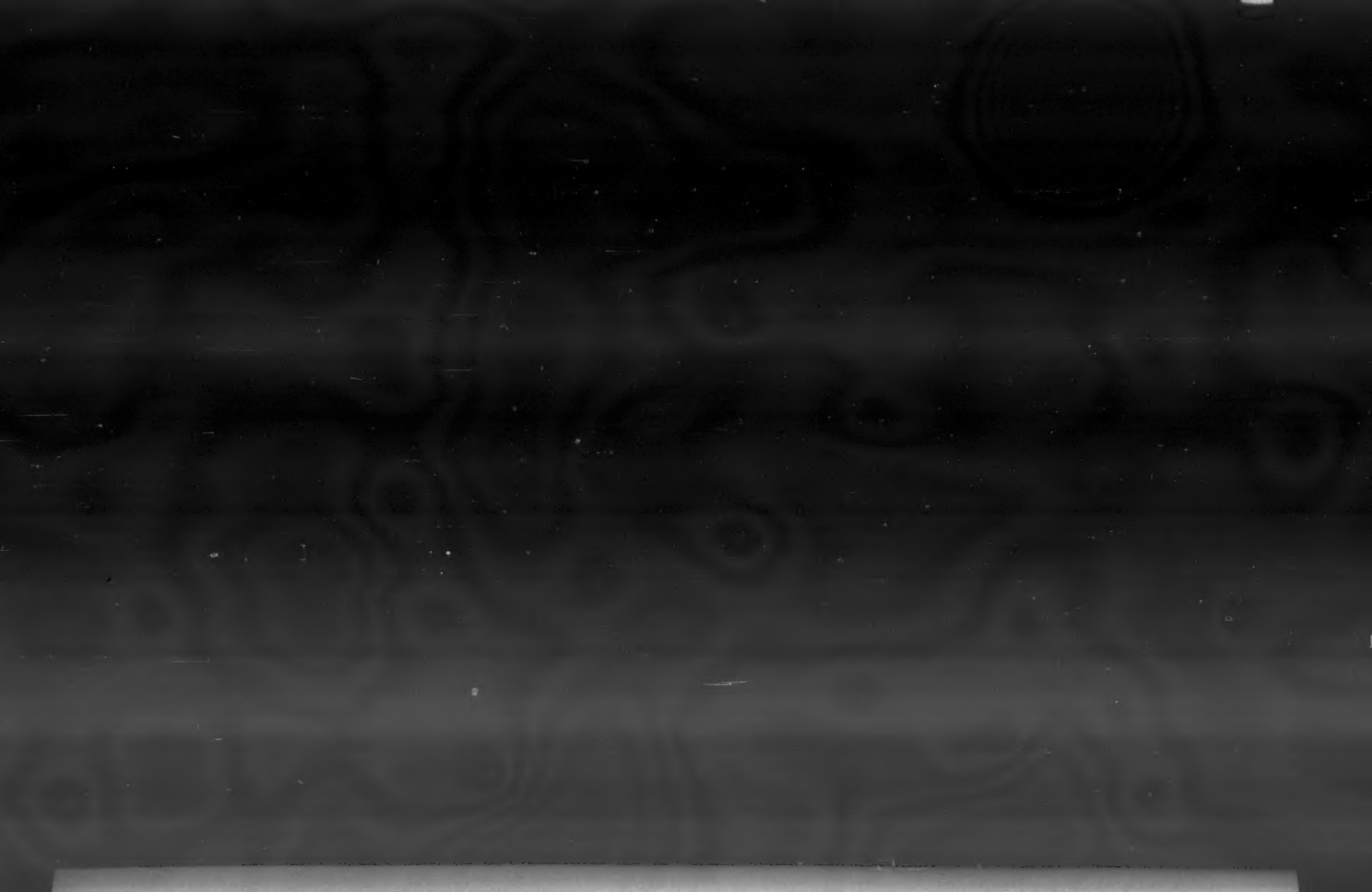
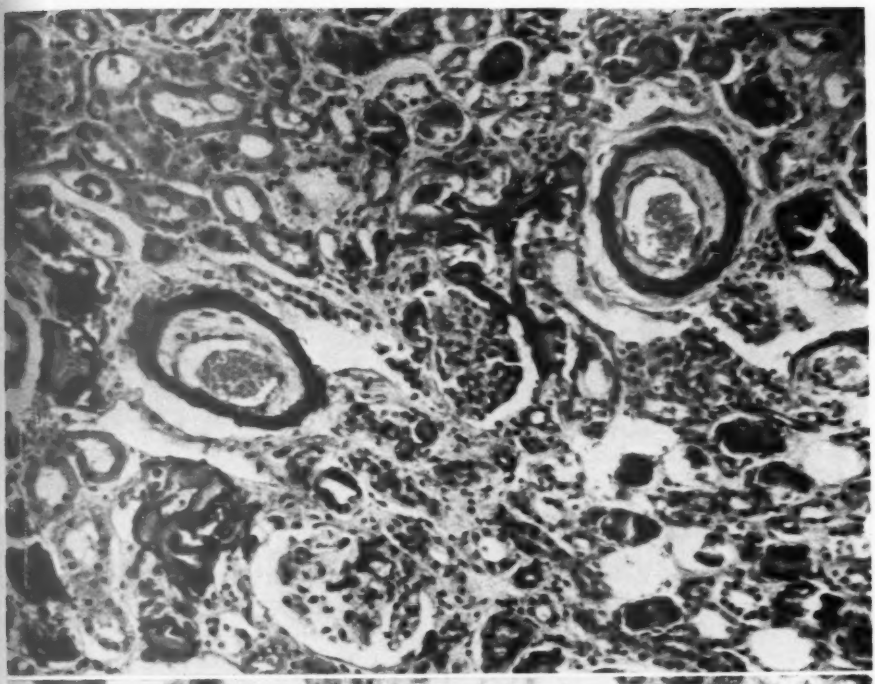
FIG. 9. Renal cortex in a rabbit given 100,000 units of viosterol daily for 24 days followed by a normal regime for 21 days. There is widespread degeneration with calcification of tubules, glomeruli and arteries. The two medium-sized arteries illustrate a high degree of medial calcification, a secondary fibrocellular intimal proliferation with narrowing of the vascular channels and the early stages of avascular resorption of calcified medial structure as indicated by the irregular contour of the margins of calcific deposits in each artery. × 160.

FIG. 10. Renal cortex in a rabbit given 100,000 units of viosterol 5 times each week for 4 weeks. The tubules are normal. The glomerulus is adherent by capsular proliferation to a degenerated calcified zone of Bowman's membrane. The afferent arteriole is heavily impregnated with calcium, as indicated by the dark homogeneous stain with hematoxylin. × 380.





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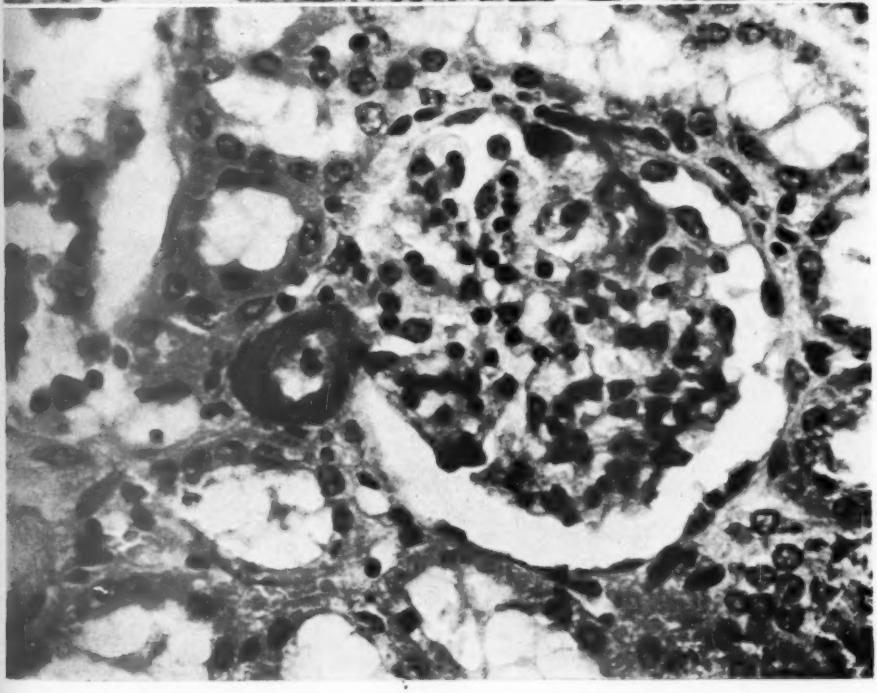

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STUDIES ON EXPERIMENTAL SHOCK: PRODUCTION OF ISCHEMIC NECROSIS OF THE SKIN BY AN INTRADERMAL INJECTION OF ENDOTOXIN OR VASOPRESSOR AMINE

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In 1928 Shwartzman[1] reported the production of cutaneous hemorrhagic necrosis in rabbits by an intradermal and an intravenous injection of bacterial filtrates. Since that time, it has been shown that a number of substances, when injected intradermally, are capable of inducing similar lesions if given in association with an intravenous injection of gram-negative endotoxin.[2,3] It has been demonstrated also that the administration of substances such as heparin[4] or nitrogen mustard[5,6] inhibit the development of the dermal lesion.

In 1956 Thomas[7] reported that dermal hemorrhagic lesions which resembled the cutaneous Shwartzman lesion could be produced in rabbits by an intravenous injection of endotoxin followed by an intradermal injection of epinephrine or levarterenol or by an intradermal injection of a mixture of endotoxin and epinephrine. Zweifach, Nagler, and Thomas[8] described also an altered state of reactivity to epinephrine in the vessels of the rat meso-appendix when this material was applied topically after an intravenous injection of endotoxin. The results of these studies led these investigators to postulate that endotoxin altered the reactions of blood vessels to epinephrine in such a manner that the hormone became a capable necrotizing agent.

In 1958 Gatling[9] reported the induction of hemorrhagic skin lesions in rabbits by an intradermal injection of epinephrine or levarterenol following the intravenous administration of horse serum to which the animals had been sensitized previously. This investigator postulated that circulating antigen and its antibody were necessary for the production of the alteration, and he presented data identifying the lesion with that of the Arthus phenomenon. In 1959 Anderson and Brunson[10] described certain lesions produced in rabbits subjected to acute rotational shock. They reported that the lethal outcome and the incidence and severity of the

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lesions were increased greatly if the shock procedure was carried out in conjunction with an intravenous injection of endotoxin. It was suggested that these effects were mediated through adrenal medullary hormones.

The present paper reports the results of a series of experiments in which cutaneous necrosis developed in rabbits given an intradermal injection of endotoxin or a vasopressor amine in conjunction with rotational shock. These lesions, while similar in some respects to those described by Thomas [7] and Gatling,[9] exhibited striking differences in other respects.

Material and Methods

Hybrid albino rabbits of both sexes, weighing approximately 1.5 kg., were used in the study. They were fed Purina Rabbit Pellets and given free access to water.

The animals were subjected to a 15-minute period of rotation in a Noble-Collip drum [11] (450 revolutions), modified to the extent that it had no baffles and was padded with foam rubber to reduce trauma. This duration of rotation, as described previously,[10] produced a state of marked prostration and shock in the animals. In conjunction with rotation in the drum, the animals were given an intradermal injection of endotoxin or a sympathomimetic amine. For this purpose the abdominal hair was depilated, using the method described by Pitesky and Last.[12] No significant differences were observed in the dermal lesions in those depilated before and those depilated after rotation.

In initial studies, endotoxin, epinephrine, and levarterenol were used. The endotoxin consisted of the lipopolysaccharide fraction derived from *Escherichia coli* (0:111 B4) obtained from Difco Laboratories, Detroit, Michigan. Dilutions were made in sterile, isotonic, pyrogen-free saline solution and given in an amount of 100 μg. in a volume of 0.2 ml. The epinephrine and levarterenol were given in dosages of 100 μg. in a volume of 0.1 ml. In later studies a variety of other substances was used. These included phenylephrine (Neo-Synephrine ®), ephedrine sulfate, isopropylarterenol (Isuprel ®), heparin, nitrogen mustard, and certain phenothiazine derivatives. The ephedrine was injected in a dosage of 100 μg. in 0.5 ml., phenylephrine in a dosage of 100 μg. in 0.1 ml., and isopropylarterenol in a dosage of 10 μg. in 1.0 ml.

Heparin sodium, in a concentration of 10 mg. per ml., was given intravenously in a dosage of 20 mg. immediately prior to drum rotation and intradermal injections of 100 μg. of endotoxin and epinephrine. In other animals an intravenous injection of nitrogen mustard was given in a dosage of 1.5 mg. per kg., 3 days prior to intradermal injections of endotoxin and epinephrine in conjunction with drum rotation. An additional group of rabbits was given an intravenous injection of 2.5 mg. each of promethazine (Phenergan®) and promazine (Sparine®) mixture. This mixture was used because it had been shown to modify the pressor responses to epinephrine and levarterenol.[13] Following administration of the mixture, the animals were given an intradermal injection of endotoxin and levarterenol, and subjected to drum shock.

In a majority of the animals, two test substances were injected at separate sites on the abdomen. Two groups of control animals were used: one group was given intradermal injections of the test substances without rotation, and the other was given an intradermal injection of 0.2 ml. of sterile isotonic saline solution in conjunction with drum rotation. Details concerning the numbers of animals used in the various experiments are summarized in the text.

Results

The results obtained by an intradermal injection of gram-negative endotoxin, epinephrine, or levarterenol are summarized in Table I. The

figures in the table include only those animals that survived longer than 4 hours after rotation. A high percentage of the animals given the test substances simultaneously with the onset of rotation developed cutaneous necrosis. No lesions developed in control animals given only intradermal endotoxin, epinephrine, or levarterenol, or in those given intradermal saline simultaneously with drum rotation.

TABLE I

INCIDENCE OF CUTANEOUS NECROSIS IN RABBITS GIVEN INTRADERMAL INJECTIONS OF ENDOTOXIN, EPINEPHRINE, OR LEVARTERENOL SIMULTANEOUSLY WITH ROTATIONAL SHOCK

Material injected	No. of animals	Dermal necrosis
Endotoxin	30	21 (70%)
Epinephrine	14	10 (71%)
Levarterenol	15	9 (60%)
Controls *	12	0 (0%)

* See text for details.

The lesions were similar in all groups of animals, and were evident on inspection within 4 hours after rotation. They were characterized by a localized area of ischemia about the injection site in which the skin was a pearly white color. Over the next few hours the skin became dry, brown, and parchment-like. The area of involvement extended from the injection site for varied distances, and invariably was greater in those given epinephrine (Figs. 1, 3, 4 and 7) of levarternol (Fig. 5) than in those given endotoxin (Fig. 2). Dilatation of superficial blood vessels over the area was noted occasionally, but frank hemorrhage in or around the lesion was never observed. In the following 18 to 24 hours the skin became progressively drier and more brittle, and cracks or fissures appeared (Figs. 1 and 2). There was little evidence of edema, however. Within 72 hours, areas of eschar formation appeared and fragments of the dried skin began to drop off (Fig. 3). In 96 hours, extensive eschar formation was present and desquamation occurred (Figs. 4 and 5).

To determine the role of ischemia in causation of the lesions, the animals were given an intravenous injection of fluorescein (2 ml. of a 2 per cent solution) at varied intervals after the intradermal injections, and the abdomens were examined under ultraviolet light (Wood's lamp) for evidence of fluorescence. In those animals which had been given epinephrine or levarterenol, nonfluorescence, frequently over as much as 50 per cent of the abdominal skin area, was noted for as long as 6 hours after intradermal injection. On the contrary, in those given endotoxin, the period of ischemia and nonfluorescence diminished by the end of 2 to 3 hours, and at 6 hours the injection site was marked by high intensity fluorescence.

Microscopic examination of the skin in animals that died or were sacrificed at varied periods after intradermal injection and rotation showed similar alterations in all groups, with one exception. Early changes were characterized by complete ischemia of the skin and subcutaneous tissues, with necrosis of the superficial portions of the epi-

TABLE II

EFFECT ON DERMAL NECROSIS OF VARIATIONS IN THE INTERVAL BETWEEN INTRADERMAL INJECTION AND ROTATION

Procedure	Material injected	No. of animals	Dermal necrosis
Injection 4 hours prior to rotation	Endotoxin	8	1 (13%)
	Epinephrine	9	2 (22%)
	Levarterenol	3	0 (0%)
Rotation 4 hours prior to injection	Endotoxin	7	5 (71%)
	Epinephrine	4	3 (75%)
	Levarterenol	3	2 (66%)

dermis. At 18 to 24 hours this was marked by a heavy crust composed of necrotic squamous cells, nuclear debris, and minimal amounts of fibrin and red cells (Figs. 9 to 11). These lesions persisted for as long as 5 days. Rarely were occluded blood vessels observed, and the arterial walls displayed no evidence of necrosis. In all instances there was a striking absence of hemorrhage.

In the lesions produced by endotoxin, varying degrees of inflammatory reaction composed of heterophils and mononuclear cells were observed. In some, the reaction was quite sparse (Fig. 9), but in others it was severe and extended into the underlying connective tissue (Figs. 6 and 7). In those lesions produced by the vasopressor substances, a paucity of inflammatory cellular reaction was noted in lesions examined as long as 3 to 5 days after rotation (Figs. 8 and 10). Sections of skin from animals of both groups at this time showed extensive deposits of subepidermal hyaline material and varying degrees of connective tissue proliferation (Figs. 10 and 12). One other change, common to both groups, consisted of extensive foci of muscle calcification unaccompanied by any cellular reaction.

It has been reported that the systemic effects produced by drum rotation and intravenous injections of endotoxin are dependent on the interval between rotation and the administration of endotoxin.[10] To investigate the effect of time variation, endotoxin, epinephrine, and levarterenol, in the same dosages and volumes as described previously, were administered to one series of animals 4 hours before and to another series 4 hours after drum rotation. As may be seen in Table II, there was a low incidence

of lesions when the test substances were injected 4 hours before rotation. Only 1 of 8 animals given endotoxin, and only 2 of 9 animals given epinephrine developed cutaneous ischemic necrosis; the size and extent of these lesions was considerably less than that described in the previous groups of animals. When the injections were carried out 4 hours after rotation, however, a high percentage of the animals developed cutaneous necrosis. These lesions were similar in incidence and severity to those which occurred following intradermal injections of these substances simultaneously with rotation (Table I).

In the studies described by Thomas[7] and Gatling,[9] dermal necrosis failed to develop in rabbits given ephedrine, suggesting that vasoconstriction *per se* was not an important factor in the production of the hemorrhagic lesions. The effects of other vasopressor amines on the development of dermal lesions in rabbits subjected to rotational shock, are summarized in Table III. As may be seen, a high percentage of the animals in each group developed dermal lesions. These were similar in appearance and severity to those produced by endotoxin, epinephrine, or levarterenol.

It has been shown that the dermal Shwartzman phenomenon is inhibited by heparin[4] or nitrogen mustard,[5,6] but that the administration of chlorpromazine, a phenothiazine derivative, does not influence its development.[7] Thomas[7] reported that the epinephrine-endotoxin dermal lesions were prevented by the administration of chlorpromazine, but were enhanced by heparin or nitrogen mustard. Gatling,[9] on the other hand, reported that heparin "ameliorated" but did not prevent hemorrhagic dermal necrosis in his experiments. To test the effects of these substances on the drum-induced dermal lesions described previously, experiments were carried out in which heparin, nitrogen mustard, or a mixture of

TABLE III

INCIDENCE OF CUTANEOUS NECROSIS IN RABBITS GIVEN INTRADERMAL INJECTIONS OF VARIOUS SYMPATHOMIMETIC AGENTS SIMULTANEOUSLY WITH ROTATIONAL SHOCK

Material injected	No. of animals	Dermal necrosis
Phenylephrine	6	5 (83%)
Ephedrine	8	4 (50%)
Isopropylarterenol	4	4 (100%)

promazine-promethazine was given to rabbits subjected to drum shock simultaneously with an intradermal injection of endotoxin, epinephrine, or levarterenol.

The results, summarized in Table IV, show that neither heparin nor nitrogen mustard, in the dosages used, prevented the development of the

dermal lesions, which occurred in all of the animals given either endotoxin or epinephrine. On the contrary, the lesions were either prevented or markedly attenuated by prior administration of the mixture of promethazine-promazine. Only one of the animals in each group developed ischemic dermal necrosis (Table IV), and this was considerably

TABLE IV

EFFECT OF HEPARIN, NITROGEN MUSTARD, OR PHENOTHIAZINES ON THE PRODUCTION OF DERMAL LESIONS

Intravenous injection *	Intradermal injection	No. of animals	Dermal necrosis
Heparin	Endotoxin	4	4 (100%)
	Epinephrine	4	4 (100%)
Nitrogen mustard	Endotoxin	4	4 (100%)
	Epinephrine	4	4 (100%)
Phenothiazine	Endotoxin	5	1 (20%)
	Levarterenol	4	1 (25%)

* Details of administration in text.

less severe and more localized than the lesions in animals given endotoxin or levarterenol alone in conjunction with drum rotation.

DISCUSSION

A previous report [10] described the systemic lesions induced in rabbits by acute rotational shock alone and in conjunction with an intravenous injection of gram-negative endotoxin. The effects in those subjected to rotation alone were similar to those in rabbits following a single intravenous injection of endotoxin, suggesting that the rotational procedure acted in a manner basically similar to that of endotoxin. The acute stress procedure, undoubtedly accompanied by an increased secretion from the adrenal gland, led the authors to postulate that adrenal medullary hormones in some manner participated in the actions of endotoxin, or that the actions of endotoxin were mediated through adrenal medullary hormones.

The occurrence of ischemic and hemorrhagic intestinal lesions in rabbits subjected to drum rotation suggested also that there might be absorption into the blood stream of either gram-negative micro-organisms, or products thereof (lipopolysaccharides) which could contribute to the development of systemic lesions. Thus, it seemed plausible to believe that drum-shocked animals might have not only increased quantities of adrenal hormones in their circulatory system, but also, perhaps simultaneously, endotoxin or endotoxin-like substances derived from the intestinal flora. The experiments described by Thomas,[7] in which dermal

hemorrhagic necrosis was produced by an intradermal injection of epinephrine in rabbits given intravenous endotoxin, or by an intradermal injection of a mixture of endotoxin and epinephrine, suggested an ideal method for testing the hypotheses mentioned above. Consequently, the series of experiments described here were performed.

The results of these studies show that cutaneous necrosis may, indeed, be induced in shocked rabbits by the intradermal injection of epinephrine (or levarterenol) or endotoxin. Although the method used is similar to that used by Thomas [7] and Gatling,[9] the resulting lesions bear little gross or microscopic similarity to the lesions reported by these investigators. The differences warrant consideration. The lesions described in these experiments seem to be caused primarily by a prolonged interval of ischemia. Evidence for this fact is their gross appearance, the absence of fluorescein penetration into the injected sites for extended periods of time, and absence of dilated vessels, petechiae, or hemorrhage. Histologically also, the lesions are characteristic of ischemic necrosis. Secondly, the lesions resolve by desquamation of the skin in and about the sites of injection; this is associated with subepidermal accumulations of hyaline material and connective tissue proliferation. They do not, in contrast to the lesions described by Thomas, "gradually fade in color." [7] Thirdly, it should be emphasized that similar dermal lesions were produced by injections of other vasopressor amines such as ephedrine, isopropylarterenol, or phenylephrine. In this respect they differ sharply from those reported previously, since neither Gatling [9] nor Thomas [7] was successful in producing dermal hemorrhagic necrosis by the administration of such substances as ephedrine.

The epinephrine (or other vasopressor) lesion observed in this investigation is similar to the lesions described by Thomas in its striking absence of inflammatory reaction. On the other hand, the lesion induced by endotoxin resembles that induced by the intradermal injections of mixtures of epinephrine-endotoxin,[7] in that there was often a heavy infiltrate of inflammatory cells. The lesions were similar also to those of Thomas in that they exhibited no evidence of vascular occlusion or vascular fibrinoid deposition. A comparison of the various features is shown in Table V.

The influence of several modifying agents on the production of dermal necrosis is summarized in Table VI. The lesions observed in the present series of experiments resembled more closely those of Thomas [7] than of other investigators, in that they were prevented by the administration of phenothiazine derivatives (chlorpromazine or promazine-promethazine), and were either unaltered or enhanced by administration of heparin or nitrogen mustard. In an attempt to study the effects of cortisone on the

TABLE V

COMPARISON OF THE FEATURES OF CUTANEOUS LESIONS REPORTED BY VARIOUS AUTHORS

Author or reaction	Gross features					Microscopic features			Fate of lesion
	Ischemia or pallor	Petechiae	Hemorrhage	Edema	Induration	Vascular lesions	Hemorrhage	Cellular infiltrate	
Shwartzman [1]	Transient	+	+	±	−	+	+	+	Fades
Thomas [7]	Transient *	+	+	−	−	±	+	−	Fades
	Transient †	+	+	+	+	±	+	+	Fades
Gatling [9]	Transient	+	+	+	+	+	+	+	Fades
Evers & Brunson	Persistent	−	−	−	−	±	−	+ (endotoxin) − (vasopressor)	Desquamates

* Reaction produced by intradermal epinephrine or endotoxin after an intravenous injection of endotoxin.

† Reaction produced by intradermal injection of mixture of endotoxin and epinephrine.

shock-induced dermal lesions, rabbits were pretreated for 3 days with cortisone acetate, following which they were subjected to rotation in conjunction with intradermal injections of epinephrine or endotoxin. Unfortunately, it was not possible to ascertain definitive results, since all animals so treated died during the period of rotation.[14]

TABLE VI

COMPARISON OF MODIFYING ACTION OF VARIOUS SUBSTANCES ON CUTANEOUS LESIONS

Author or reaction	Modification of lesion			
	Heparin	Cortisone	Phenothiazine	Nitrogen mustard
Shwartzman[1]	Prevents	Enhances	No effect	Prevents
Thomas[7]	Enhances	Prevents	Prevents	Enhances
Gatling[9]	Ameliorates	?	No effect	?
Evers & Brunson	No effect	?*	Prevents	Enhances

* All animals died within 4 hours after pretreatment with cortisone and rotation in drum.

From these observations and other data now extant, it appears reasonable to conclude that vasospasm, with prolonged dermal ischemia, plays a major role in the development of the lesions in this series of experiments. This period of ischemia appears to be particularly severe in the vasopressor induced lesions, to such an extent that an inflammatory reaction is inhibited, but of less severity and shorter duration in the endotoxin-induced lesions. Further, inhibition of vasospasm by agents known to exert adrenolytic effects, such as the phenothiazine derivatives, prevented the development of the lesions. On the contrary, agents such as heparin had no effect on their development, indicating that vascular thrombosis and hemorrhage were relatively unimportant in this regard. It is difficult, however, to assess the role of nitrogen mustard, since its administration seemed to enhance the development of the lesions.

That vasospasm may not be the only factor, however, is shown by other studies in which rabbits were given repeated intradermal injections of epinephrine alone, without developing cutaneous necrosis.[7] Similarly, certain other substances such as normal rabbit plasma or old tuberculin, given intradermally just prior to drum rotation, have been shown to induce lesions comparable in all respects to those described here.[14] Furthermore, it has not been possible to induce cutaneous lesions in rabbits given intradermal epinephrine or endotoxin during hemorrhagic shock,[15] a state which is usually accompanied (at least transiently) by peripheral vasoconstriction.

These observations and the recorded differences in the dermal lesions, suggest two hypotheses: (1) The shock state induced by drum rotation differs in its pathogenesis from that induced by endotoxin or hemorrhage.

(2) Certain other, endogenous agents or factors, in addition to those injected intradermally, participate in the production of dermal lesions in rabbits subjected to drum shock. The nature and origin of these factors, if such exist, and the mechanisms involved in the production of rotational shock remain obscure, and their elucidation requires further investigation.

Summary and Conclusions

Ischemic cutaneous necrosis was produced in rabbits subjected to rotational shock in conjunction with an intradermal injection of epinephrine, levarterenol, or endotoxin. The incidence of the lesions was approximately 70 per cent when the test substances were given simultaneously with or 4 hours after drum rotation, but was less than 25 per cent when they were administered 4 hours prior to rotation.

The lesions were evident grossly within 4 hours after rotation. They were characterized by extensive pale, flat ischemic areas which progressed rapidly to desiccation and, later, to desquamation. The histologic features consisted of necrosis of the superficial epidermis with a paucity of inflammatory reaction except in the endotoxin lesions. Vascular occlusion was significantly absent. Lesions observed 3 to 5 days after rotation consisted of extensive accumulations of subepidermal hyaline substance and connective tissue proliferation. Similar dermal lesions were induced by rotational shock in association with the intradermal injection of ephedrine, phenylephrine, or isopropylarterenol.

The lesions were prevented by the intravenous administration of a mixture of promazine and promethazine, but were unaffected by the prior administration of heparin. Pretreatment with nitrogen mustard appeared to increase the severity of the lesions.

The pathogenesis of the dermal lesions, and their similarity to those described by other authors, are discussed. It is concluded that vasospasm, although playing a major role in the causation, is probably not the only factor involved.

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[*Illustrations follow*]

Legends for Figures

All microscopic sections were stained with hematoxylin and eosin.

Fig. 1. Abdomen of a rabbit given epinephrine intradermally immediately prior to rotation; photographed 24 hours after rotation. Note the eschar in the central portion of the lesion.

Fig. 2. Abdominal lesion at the site of intradermally injected endotoxin 24 hours after rotation. The lesion is similar to that in Figure 1, but is much less extensive.

Fig. 3. Abdominal lesion at the site of intradermally injected epinephrine 72 hours after rotation. Note progression of the lesion as compared to that in Figure 1.

Fig. 4. Abdominal lesion at the site of intradermally injected epinephrine 96 hours after rotation. Note extensive eschar formation and beginning desquamation.

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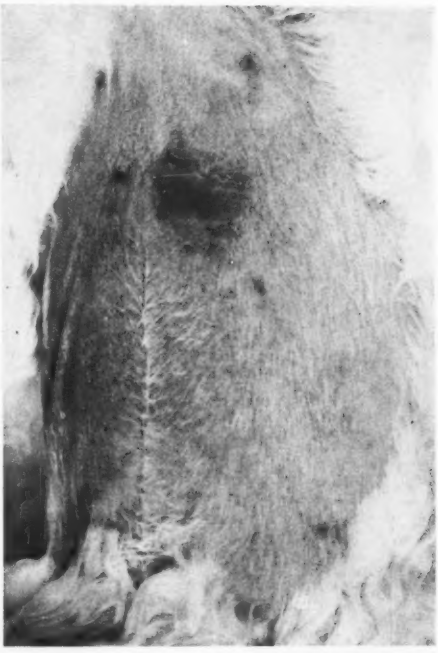

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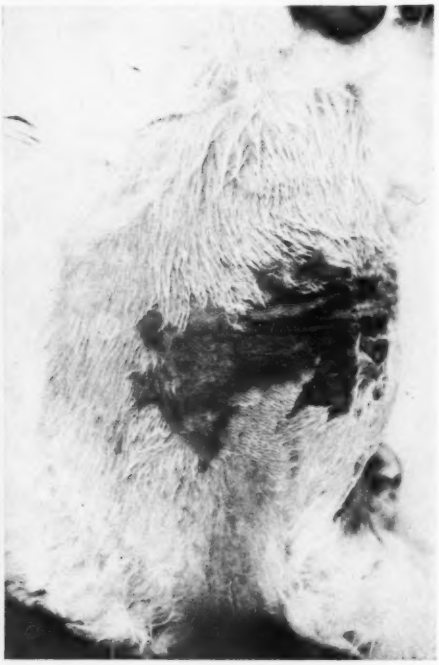

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FIG. 5. Abdominal lesion at the site of intradermally injected levarterenol 96 hours after rotation. The changes are similar to those shown in Figure 4.

FIG. 6. A dermal lesion in an animal receiving an injection of endotoxin immediately prior to rotation and sacrificed 3 days later. Note the epidermal and dermal necrosis with extensive cellular reaction composed predominantly of heterophils and a few mononuclear cells. These extend through all layers of the skin into the muscle. There is beginning subepidermal hyalinization. × 40.

FIG. 7. Another animal treated as indicated in Figure 6; section taken at 3 days. Ischemic necrosis is evident in all layers of the skin, with infiltration by heterophils into underlying tissue and muscle. There is also muscle necrosis. × 40.

FIG. 8. An intradermal epinephrine injection site; section taken 3 days after rotation. Note the absence of cellular reaction or hemorrhage and the extensive ischemic necrosis. × 40.

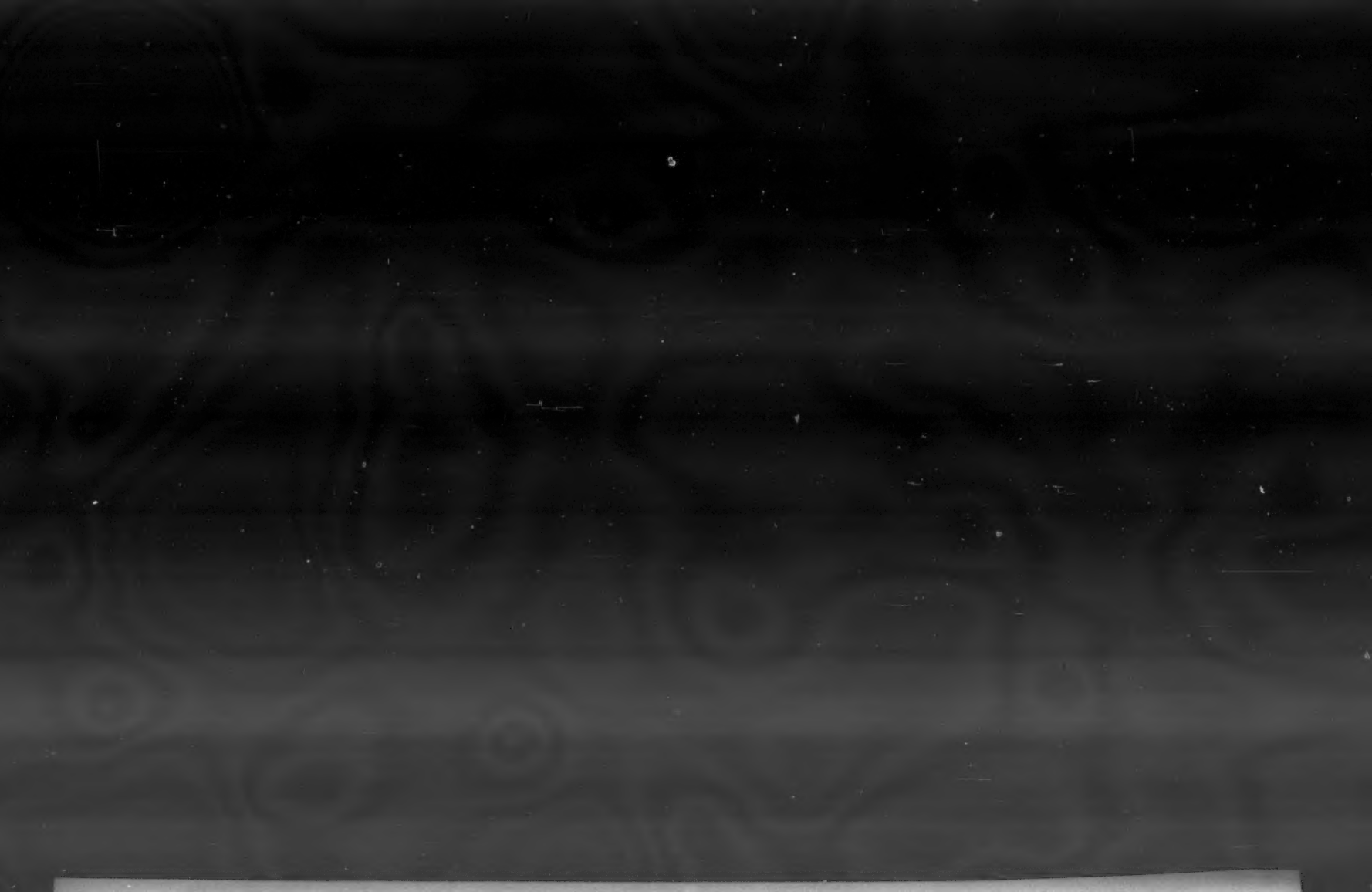

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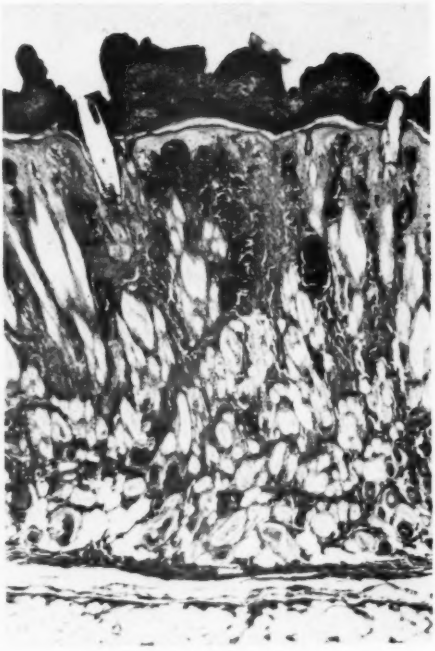
FIG. 9. An intradermal endotoxin site; section taken 4 days after rotation. The exudate is composed of nuclear debris and some red cells. There is extensive superficial necrosis and a mild underlying cellular infiltrate. × 40.

FIG. 10. An animal given intradermal levarterenol; section taken 4 days after rotation. Epidermal necrosis shows absence of cellular reaction, extensive hyalin deposition and beginning connective tissue proliferation. × 40.

FIG. 11. An intradermal phenylephrine site 4 days after rotation. Note ischemic necrosis, paucity of inflammatory cells, and absence of edema and hemorrhage. × 40.

FIG. 12. An intradermal endotoxin injection site 7 days after rotation. Note the remnant of the crust, regenerated epithelium, subepidermal hyalin deposition and extensive connective tissue proliferation. × 40.

9



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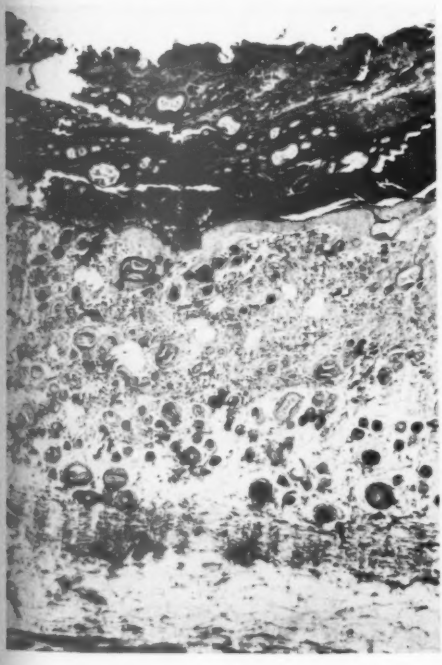

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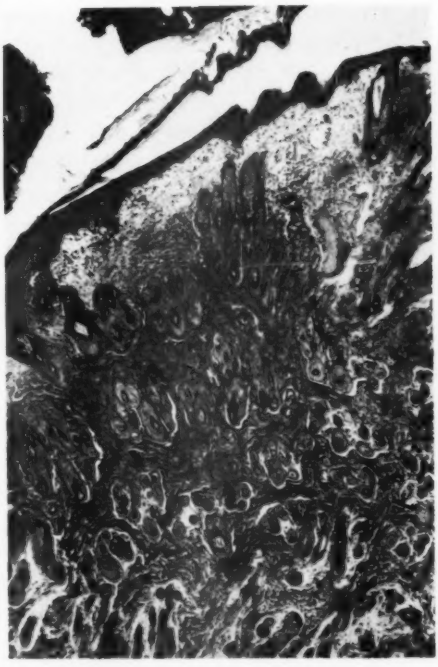

12

HYPERACTIVITY OF THE RETICULOENDOTHELIAL SYSTEM AND EXPERIMENTAL ANEMIA IN MICE

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Banti first applied the term splenic anemia to a disease characterized by anemia and splenomegaly.[1] In 1882 he formulated two possible mechanisms for its pathogenesis, namely: (a) the myelo-inhibitory and (b) the cytolytic or erythroclastic theories. Though there has been little evidence to support the former concept, the problem is not yet resolved. In addition, evidence has been presented recently to support an immunologic mechanism in some examples of human "hypersplenism."[2-4]

To further study the problem, many investigators have attempted to construct an experimental model of the condition. A syndrome similar to human hypersplenism has been produced in animals by the repeated administration of a variety of colloidal substances which, when sequestrated by the reticuloendothelial system (RES), result in a marked reactive cellular proliferation. Hueper noted that dogs treated with large doses of methylcellulose developed a moderate degree of anemia associated with a large liver and spleen.[5] Similar observations have been made following the administration of gum acacia and polyvinylpyrrolidone. Palmer, Eichwald, Cartwright and Wintrobe[6] reported that intraperitoneal injections of methylcellulose in rats resulted in massive splenomegaly associated with a mild anemia, leukopenia and thrombopenia. The hematologic manifestations were reversed or prevented by splenectomy, though the lesions produced in the liver, kidneys and bone marrow remained unchanged. Giblett and his collaborators[7] showed that the erythrocytes of the methylcellulose-treated, anemic animal had a normal life span in the normal animal, thus apparently eliminating an intrinsic corpuscular defect in the pathogenesis of the anemia.

It seemed to us that a common feature of these experimental models was a hyperplastic RES in which accelerated destruction of normal erythrocytes occurred. A study of the phagocytic function of the RES, utilizing the quantitative techniques recently described, could provide useful data concerning the pathogenesis of this anemia.[8,9]

The carbohydrate, zymosan, is a complex hemicellulose derived from

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the cell wall of yeast. It can produce marked hyperplasia and increased phagocytic activity of the RES in mice.[10] In the experiments described below, the administration of zymosan to mice was shown to produce a severe anemia associated with considerable hyperplasia of the phagocytic elements in the RES.

Material and Methods

The experiments were carried out in adult white male Swiss Webster mice. A saline suspension of zymosan (5 mg. per ml.; lot no. 7B-13, obtained from Fleischman Laboratories, Westport, Connecticut) was heated in a water bath at 100° C. for 10 minutes. To assure a homogeneous suspension, the preparation was then shaken with glass beads in a Mickle agitator for 5 minutes. Doses of 1 mg. were injected into the dorsal tail vein of each animal on alternate days for periods of 14 to 20 days. To evaluate the phagocytic function, measure the erythrocyte survival time, and study the hemogram, 0.025 ml. of blood was obtained from the retrobulbar venous plexus. No more than a total of 0.1 ml. was removed from each animal in a one-week period. On this schedule none of the control animals became anemic.

The phagocytic function of the RES was investigated by measuring the clearance from the blood of a suitable colloidal preparation of carbon particles (preparation C11 1431/a, secured from Gunther Wagner, Hanover, Germany), homogeneous in size, measuring approximately 250 Å, and suspended in gelatin. It has been shown by Biozzi, Benacerraf and Halpern that these carbon particles were selectively phagocytized by the macrophages lining the sinusoids of the liver, spleen, lymph nodes and bone marrow.[8] When such particles were injected intravenously in the correct dose range, approximately 90 per cent of the material was cleared in the liver and spleen.[8] The blood carbon concentration decreased according to an exponential function of time which was expressed as follows:

$$\log C_1 - \log C_2/T_2 - T_1 = K$$

C_1 and C_2 were the concentrations of carbon in the blood at times T_1 and T_2 respectively. K, which expressed the phagocytic activity of the RES for the injected dose, was called the phagocytic index.

It has been shown that the phagocytic index depends on the weights of the liver and spleen. These organs contain the largest proportion of the phagocytic elements, and their weights bear a third power relationship to K. This allows the use of a "corrected phagocytic index" alpha (α) which is expressed as:

$$\alpha = \frac{W}{WLS}\sqrt[3]{K}$$

W is the body weight and WLS the sum of the weights of the liver and spleen. A dose of 16 mg. of carbon per 100 mg. of body weight was employed in these experiments. The measurements of carbon clearance were carried out as described.[8]

The erythrocyte survival time was determined according to a standard method employing sodium chromate (Cr^{51}) as the red cell tag. Approximately 10 μc. were used to tag 0.2 ml. of blood in acid-citrate dextrose solution. Each animal received its own tagged erythrocytes to prevent any possible incompatibility. At regular intervals, 0.025 ml. samples of blood were removed and hemolyzed in 2 ml. of 0.1 per cent of sodium carbonate. The radioactivity was measured in a well type scintillation counter and expressed as the log of the percentage of radioactivity. The amount of radioactivity in the blood, 24 hours after the injection of tagged cells (DO) was taken as the 100 per cent value.

In order to analyze for the possible presence of adsorbed antibody on the surface

of the erythrocytes of anemic animals, an anti-mouse gamma globulin serum was prepared in rabbits. Mouse gamma globulin was prepared by salting out with 30 per cent saturated ammonium sulfate. After dialysis against normal saline, the globulin was alum-precipitated [21] and resuspended in saline. The final suspension contained 7.8 mg. of protein per ml. The material was injected into rabbits on alternate days for 2 weeks in doses of 0.5 ml., and at the end of the third week the animals were bled. The antiserum was adsorbed with normal mouse erythrocytes. Its effectiveness was indicated by its ability to agglutinate normal mouse tanned erythrocytes coated with mouse gamma globulin in high dilution.

The presence of an antizymosan antibody in the serum of treated mice was investigated with two techniques: simple agglutination of zymosan by immune serum, and immune adherence in a system with human red cells and guinea pig complement. In these experiments zymosan was used in a concentration of 500 μg. per ml. of normal saline and prepared in the manner described above. In the zymosan agglutination procedure, 0.2 ml. of this suspension was added to 0.2 ml. of the serially diluted serum to be tested. After incubation at 37° C. for 30 minutes, the tubes were placed in the cold at 6° C. overnight and examined the next morning. The reaction was considered positive if a clump of coarse flakes formed which did not resuspend easily with agitation. Immune adherence was demonstrated in a system employing zymosan, immune serum, human red cells and guinea pig complement. The procedure followed was similar to that employed by Nelson.[28]

The passive cutaneous anaphylaxis skin test was performed according to the method described by Ovary.[25]

The role of the spleen in the pathogenesis of the anemia was investigated by using splenectomized animals. Splenectomized and nonsplenectomized animals were treated with a 10 mg. course of zymosan over a 20-day period.

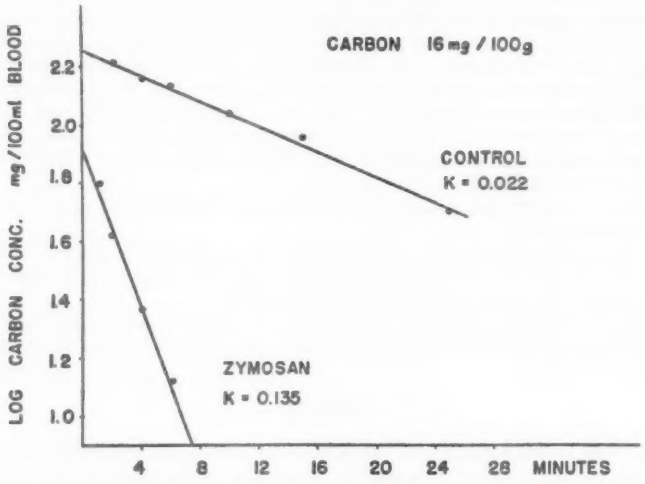
Tissues for histologic examination were fixed in Zenker's acetic solution and embedded in paraffin. Sections were prepared with Giemsa, hematoxylin and eosin, and Prussian blue stains.

Results

The phagocytic activity of the RES was greatly enhanced by repeated intravenous injections of zymosan. The rate of carbon clearance in the treated animals was approximately 6 times that of the control group. Typical curves of carbon clearance are shown in Text-figure 1. This effect was most marked after 3 one-mg. injections and was associated with a considerable increase in the weights of the liver and spleen. The initial weight increase of these organs after 2 to 4 one-mg. injections of zymosan was apparently due to hyperplasia of reticulum cells. This change could be seen within 1 to 2 days following a single intravenous dose (1 mg.) of zymosan. Following this phase, the absolute weights of the liver and spleen increased at a slower rate. The phagocytic index reached a maximum value after the fourth injection. At about this time reticulum cell hyperplasia was striking, and granulomas composed of these cells were seen within the sinusoids of the liver and spleen (Figs. 1 and 2). Compression atrophy of parenchymal cells was produced in the liver by these aggregates. When zymosan administration was continued, further increase in weight of the liver and spleen was associated with the infiltration of these organs by scattered mononuclear cells and the pres-

ence of foci of extramedullary hematopoiesis (Figs. 4 and 5). The mononuclear cells were first observed in significant numbers after the first 2 or 3 injections of zymosan. In the liver, they tended to localize in the periportal region (Fig. 3). Striking erythrophagocytosis was observed in the macrophage elements of the liver and spleen. Collections of red cells and red cell ghosts were found within the cytoplasm of the granulomatous aggregates of reticulum cells as well as within individual macrophages (Figs. 6 and 7).

The specimens of bone marrow examined in both the treated and con-



TEXT-FIGURE 1. Blood clearance of carbon in a control and in a zymosan-treated mouse. Dose of carbon: 16 mg. per hundred gm. of body weight.

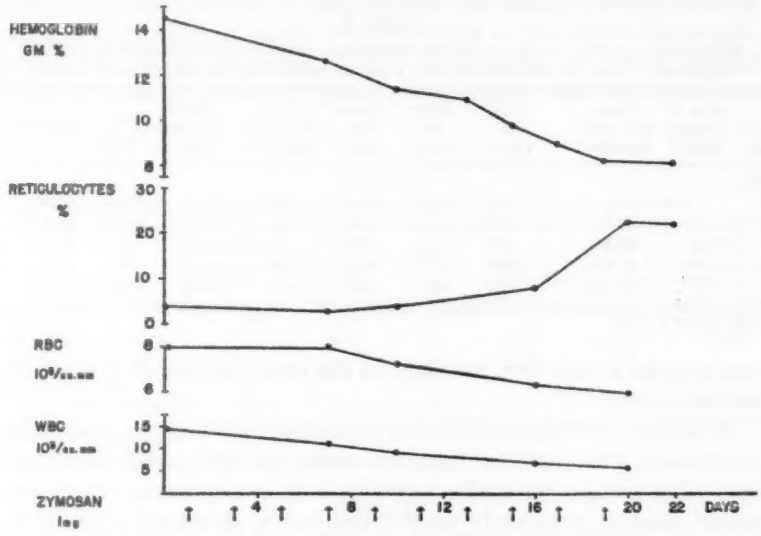
trol groups were always hyperplastic. The Prussian blue stain revealed abundant iron-containing pigment in the liver, spleen, lymph nodes and bone marrow. Scattered macrophages with foamy cytoplasm were noted in the liver, spleen and lymph nodes of the zymosan-treated animals. This cytoplasmic material stained red-violet with periodic acid-Schiff stain; however, no attempt was made to specifically identify it as zymosan.

No gross or microscopic abnormalities were observed in the other organs examined, e.g., the kidneys, adrenals, lungs and thymus gland.

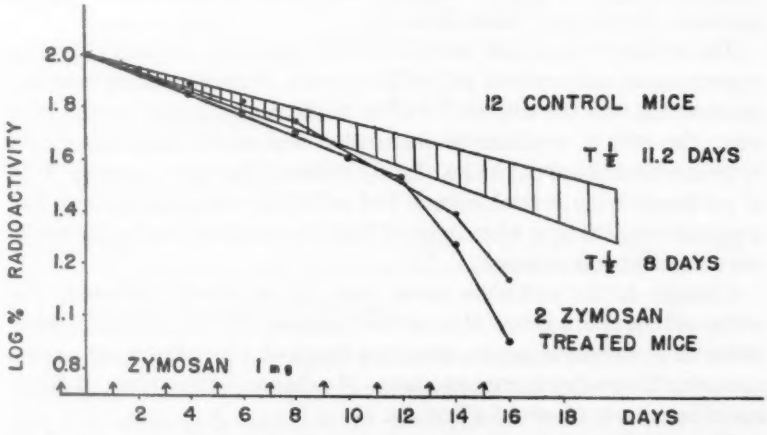
The blood hemoglobin concentration began to decrease near the end of the first week of zymosan administration and declined rapidly thereafter when treatment was continued. If zymosan was given beyond 3 weeks, many of the animals died with progressive anemia and cachexia. However, if treatment was stopped prior to the third week, virtually all of the animals recovered, and a normal hemoglobin concentration was

restored within 1 1 to 2 weeks. A sharp rise in the reticulocyte count occurred near the end of the second week (Text-fig. 2).

The decline in hemoglobin concentration was correlated in a positive manner, with a sharp reduction in erythrocyte life span. The red cell survival time, expressed in terms of the T ½ of Cr^{51}-labeled cells, was approximately 3 to 6 days in zymosan-treated mice, whereas a T ½ of



TEXT-FIGURE 2. Average hematologic values in mice treated with zymosan, 10 mg. over 20 days.



TEXT-FIGURE 3. Erythrocyte survival in normal and zymosan-treated mice measured by the fall in blood radioactivity after the injection of sodium chromate (Cr^{51}) labeled red cells.

8 to 12 days was consistently found in untreated control animals. The red cell survival time continued to decrease during zymosan treatment, as indicated by the progressive increase in slope of the curve depicting the survival time (Text-fig. 3). By the end of the second week, destruction of erythrocytes was so rapid that the animal was unable to compensate, and a severe degree of anemia developed. When zymosan treatment

TABLE I

PHAGOCYTIC ACTIVITY OF THE RETICULOENDOTHELIAL SYSTEM AFTER ZYMOSAN TREATMENT, MEASURED BY THE BLOOD CLEARANCE OF 16 MG. OF CARBON PER 100 GM. OF BODY WEIGHT

No. of mice	Amt. of zymosan (mg.)*	Time after last injection	Body wt. (gm.)	Liver wt. (gm.)	Spleen wt. (gm.)	Liver, gm./20 gm. body wt.	Spleen, gm./20 gm. body wt.	W/WLS	K	n
control										
18			30.0	1.61	0.22	1.09	0.145	15.5	0.024	4.44
4	1	48 hr.	28.8	1.76	0.30	1.22	0.209	14.2	0.038	4.52
5	3	48 hr.	30.0	2.34	0.76	1.52	0.488	9.9	0.117	4.60
15	10	48 hr.	25.0	2.29	0.64	1.82	0.510	8.6	0.115	4.20
5	8	18 days	39.2	2.44	0.55	1.24	0.280	13.4	0.028	3.99

* 1 mg. every 2 days.

was stopped at this time the slope of the curve leveled off toward the normal range.

In animals investigated following cessation of zymosan treatment, the hematologic abnormalities began to revert toward normal within 48 hours. One group of mice, after receiving 8 mg. of zymosan over a 16-day period, became moderately anemic and had a shortened erythrocyte survival time in the range of 5 to 6 days. However, normal values of RES phagocytic activity and no anemia were found 18 days after the last injection of zymosan (Table I).

The erythrocytes of the anemic animals showed a marked degree of hypochromia, anisocytosis and poikilocytosis. However, there were few spherocytes, and the osmotic fragility of the red cells was normal even when the rate of erythrocyte destruction was rapid. Red cells of the zymosan-treated animal had a slightly reduced life span (average T ½ of 7.6 days) in the normal animal. Red cells from untreated animals had a normal survival time when injected into the anemic animal after zymosan treatment was stopped.

Changes in the leukocyte count were not so closely followed. The white cell count in a group of 10 treated animals fell from an initial mean value of 15 thousand per cu. mm. to a range of 5 to 7 thousand per cu. mm. over the 20-day treatment course. No change in the white cell differential count was observed (Text-fig. 2).

The role of the spleen in the pathogenesis of the anemia was investigated by performing splenectomy in treated and untreated groups of

animals. The splenectomized animals who subsequently received zymosan developed greatly enlarged livers which were 65 per cent larger than those of the treated animals without splenectomy. The increment in phagocytic mass within the liver of the former animals appeared to exceed the portion of these cells lost by splenectomy. Splenectomy did not prevent the development of anemia in the zymosan-treated animal. The erythrocyte survival time in these animals was sharply reduced as compared with the control group. No Bartonella or Bartonella-like organisms could be demonstrated in the erythrocytes of splenectomized animals when examined on numerous occasions. The data are summarized in Table II.

TABLE II

EFFECT OF SPLENECTOMY ON THE ANEMIA PRODUCED BY ZYMOSAN TREATMENT

Mice	Initial hemoglobin (gm.)*	Final hemoglobin (gm.)*	CR^{51} T ½ days	Reticulocyte count *	Liver, gm./20 gm. body wt.	Spleen, gm./20 gm. body wt.
10 control	14.6	13.6	9.5	1.5	1.12	0.172
10 zymosan-treated †	14.7	9.1	5.5	14.4	1.69	0.464
10 splenectomized	11.9	11.9	9.4	5.1	1.57	
10 splenectomized and zymosan-treated †	13.3	6.3	4.3	11.2	2.8	

* Per hundred cc.

† 1 mg. of zymosan every other day for 20 days.

TABLE III

SEROLOGIC STUDIES ON SERUMS OF MICE TREATED WITH ZYMOSAN

Source of serums	Zymosan agglutination	Zymosan immune adherence	Anti-globulin test	PCA † zymosan
Zymosan-immunized mice (10 mg. course; 7 days after last injection)	+ 1/64	+ 1/40	Neg.	Neg.
	± 1/128			
Zymosan-immunized mice (6 mg. course; 19 days after last injection)	+ 1/256		Neg.	
Zymosan-immunized mice (7 mg. course; 2 days after last injection)	Neg.		Neg.	
Untreated mice	+ 1/4 *	+ 1/10	Neg.	Neg.

* One group.

† Passive cutaneous anaphylaxis skin test.

The results of the serologic studies are summarized in Table III. Serums of treated mice agglutinated zymosan particles in dilutions up to 1:256 while such agglutination could not be demonstrated above a titer of 1:4 in serums of control mice. Zymosan-immune adherence was demonstrated in a titer of 1:40 with the serums of one group of treated

animals whereas it was observed with the serums of the control mice in a titer of only 1:10. Such hemagglutination could be demonstrated with human red cells, and not with red cells of mice. Erythrocytes of anemic animals were not agglutinated by rabbit anti-mouse gamma globulin serum whether or not zymosan was added to the incubation mixture, thus demonstrating the absence of mouse globulin on the surface of the red cells. The passive cutaneous anaphylaxis skin test (PCA) was negative with serums from treated animals.

Discussion

As a result of the repeated administration of zymosan over a period of 20 days, Swiss mice developed profound anemia due to rapid random destruction of erythrocytes. The liver and spleen became greatly enlarged, and erythrophagocytosis was seen in these organs as well as in lymph nodes. That the animal attempted to compensate was evidenced by reticulocytosis and extramedullary hematopoiesis in the liver, spleen and lymph nodes. Anemia resulted when this mechanism proved insufficient.

The phagocytic activity of the RES was greatly increased as measured by the enhanced ability of the treated animal to clear colloidal carbon from the blood stream. Was the rapid destruction of erythrocytes solely or primarily the result of the hyperplasia and hyperactivity of the RES brought about by the administration of zymosan? Survival time of erythrocytes of anemic animals was only slightly reduced when injected into normal animals, indicating that the erythrocyte itself was not sufficiently altered to account for its destruction. The red cells of the normal animal had a normal survival time in the treated animal after zymosan administration was discontinued. It should be emphasized that when treatment was stopped, the blood picture and functional state of the RES of the anemic animal rapidly returned to normal. The continued administration of zymosan was essential for the maintenance of the anemia and increased phagocytic activity. Hence the fact that the normal red cell had a normal life span in the treated animal after zymosan treatment was stopped does not eliminate the possibility that the hyperactive RES was primarily responsible for increased erythrocyte destruction during treatment. The zymosan-treated mouse, at least in this respect, is different from the rat receiving methylcellulose injections, reported by Palmer and co-workers, in which the "hypersplenic" state, once established, maintained itself for an extended period of time.[6]

Splenectomy not only failed to prevent erythrocyte destruction but apparently was associated with an increase in severity of the anemia

in some animals. It has been demonstrated that latent infection with Bartonella or Bartonella-like organisms can become manifest in mice, following splenectomy.[14] Such a process is associated with a mild to moderate anemia. While neither Bartonella nor similar organisms were encountered, the possibility of such infection should be considered in interpreting the results obtained with splenectomy in these studies. Following splenectomy there was marked hyperplasia of the reticulum cells in the liver. The weight of this organ in these animals was 150 per cent greater than in the splenectomized, untreated animal and 65 per cent greater than in the untreated animals without splenectomy. The increment in phagocytic mass in the splenectomized group appeared to exceed the portion of cells removed with the spleen. Therefore, the fact that the splenectomized animal developed anemia does not eliminate the possibility that a hyperactive RES played an important role in the erythrocyte destruction observed.

The fact that zymosan is an antigen capable of eliciting a specific antibody response suggested the possibility that an immune mechanism might have been responsible for the destruction of erythrocytes. Zymosan or one of its breakdown products and an antipolysaccharide antibody could have adsorbed on the erythrocyte and rendered the cell acceptable to the RES. Although an immune response to zymosan does develop during the course of treatment, gamma globulin could not be demonstrated on the red cell membrane. Erythrocyte sensitization was not demonstrated after incubation with zymosan *in vitro*. Hence a direct relationship between the immune response to zymosan and the anemia observed could not be established.

Summary

Mice treated with zymosan developed a severe anemia due to increased erythrocyte destruction. This process was associated with hyperplasia and hyperfunction of the reticuloendothelial system. The state of hyperactivity and the anemia could be maintained only by the continued administration of zymosan.

Though the data are consistent with the hypothesis that anemia is the result of accelerated destruction of erythrocytes by a hyperactive reticuloendothelial system, this mechanism could not be established with absolute certainty. An immune response to zymosan develops as a result of zymosan injection. However, there is no direct evidence that these antibodies play a role in the actual destruction of the red cell. *In vitro* studies failed to demonstrate hemagglutination or hemolysis of red cells from normal or treated animals by antizymosan serum.

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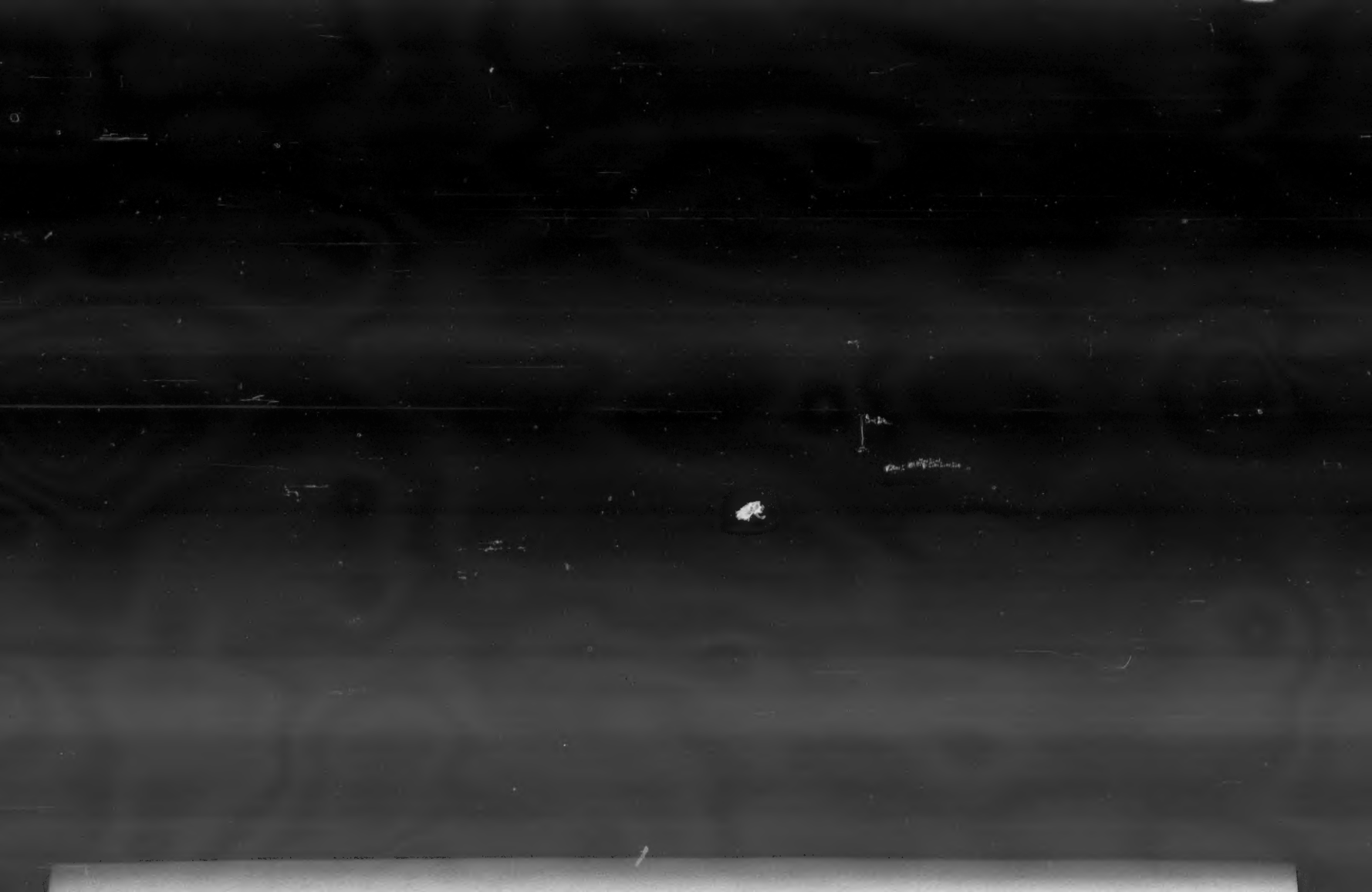
The technical assistance of Miss Doris Jacobi is acknowledged.

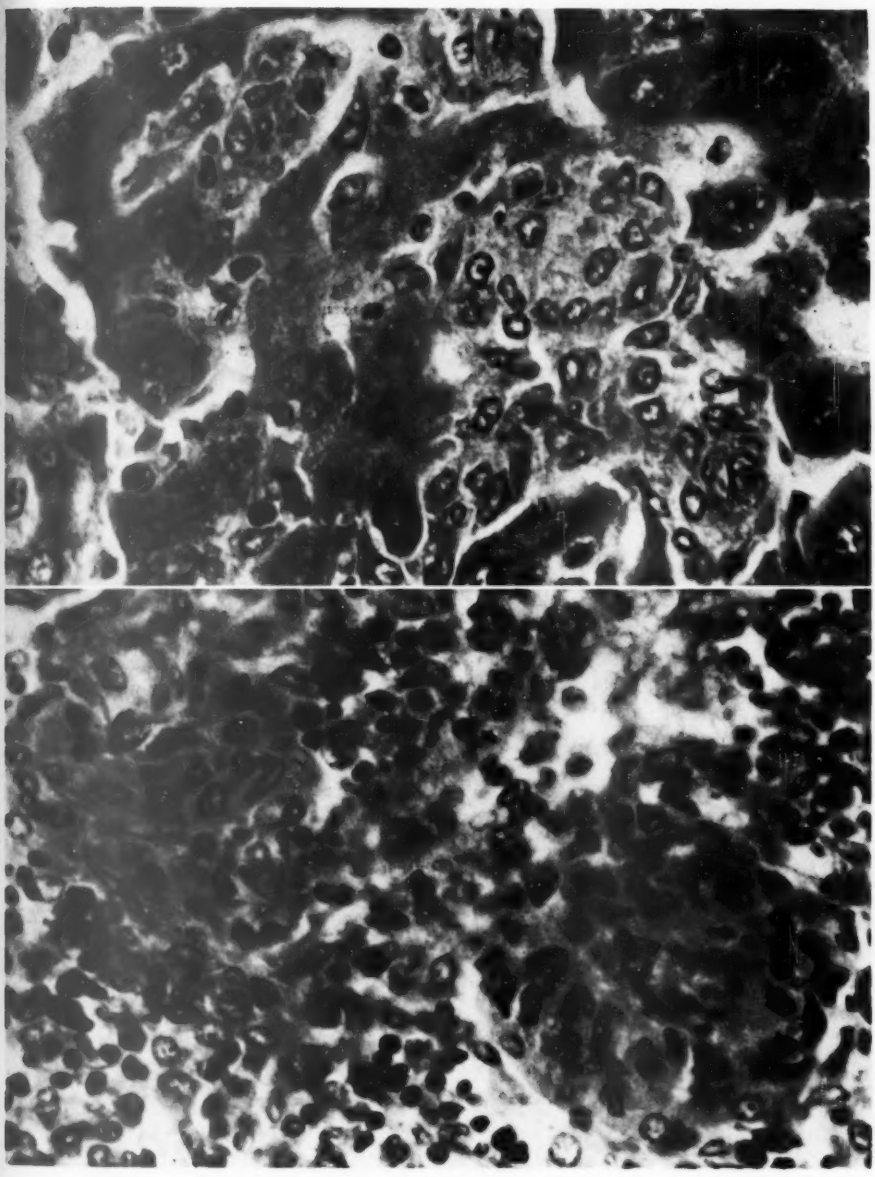
Legends for Figures

All photographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. Liver, mouse treated with zymosan (8 mg. in a 16-day period). There is striking reticulum cell proliferation. × 500.

Fig. 2. Spleen, mouse treated with zymosan (8 mg. in a 16-day period). The formation of granulomas composed of reticulum cells is evident. × 500.





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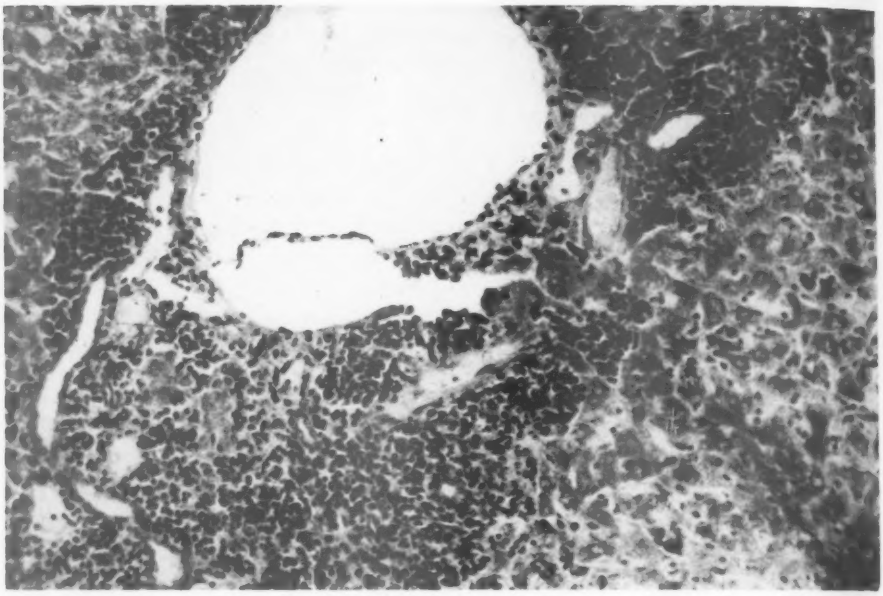
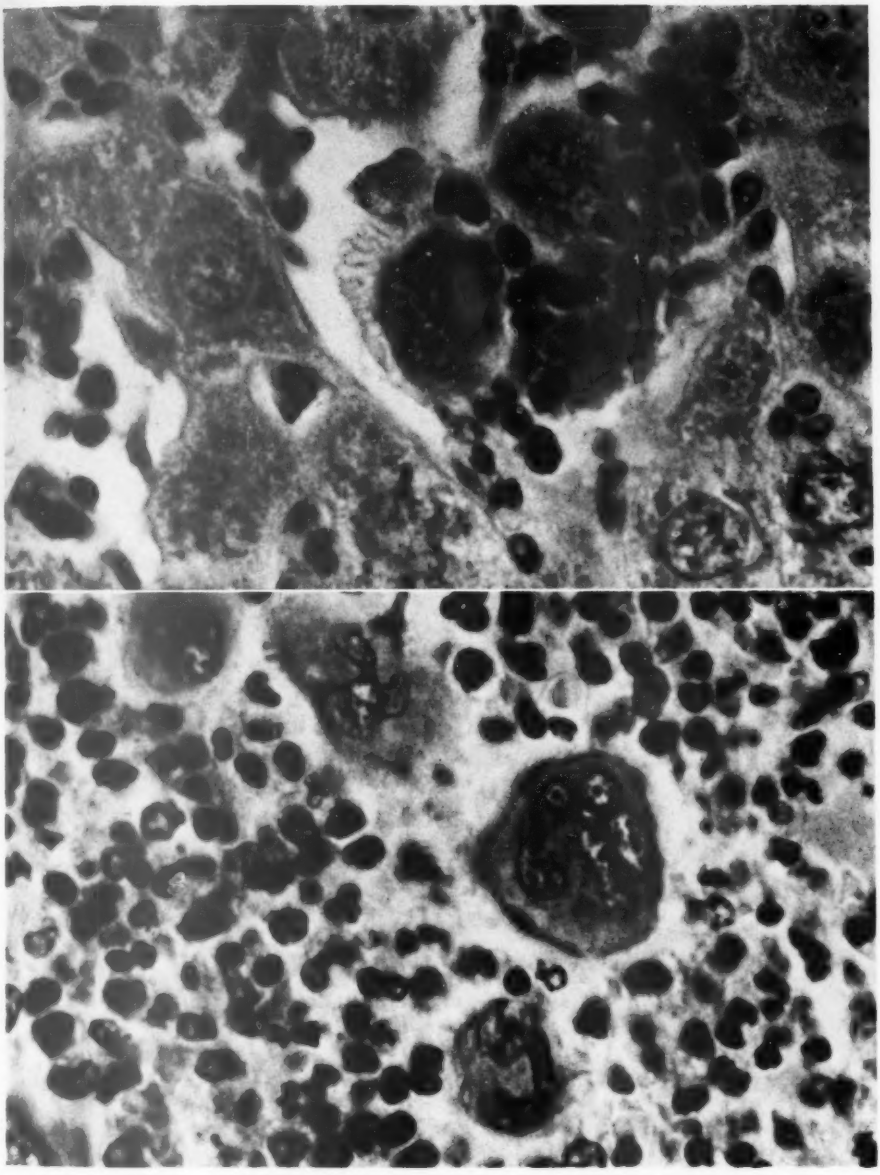


FIG. 3. Liver, mouse treated with zymosan (4 mg. over 8 days). There is infiltration of the periportal area by mononuclear cells, principally lymphocytes and plasma cells. × 120.

FIG. 4. Liver, mouse treated with zymosan (8 mg. in a 16-day period). Extramedullary hematopoiesis is shown. × 500.

FIG. 5. Spleen, mouse treated with zymosan (8 mg. in a 16-day period). Section exhibits extramedullary hematopoiesis. × 500.



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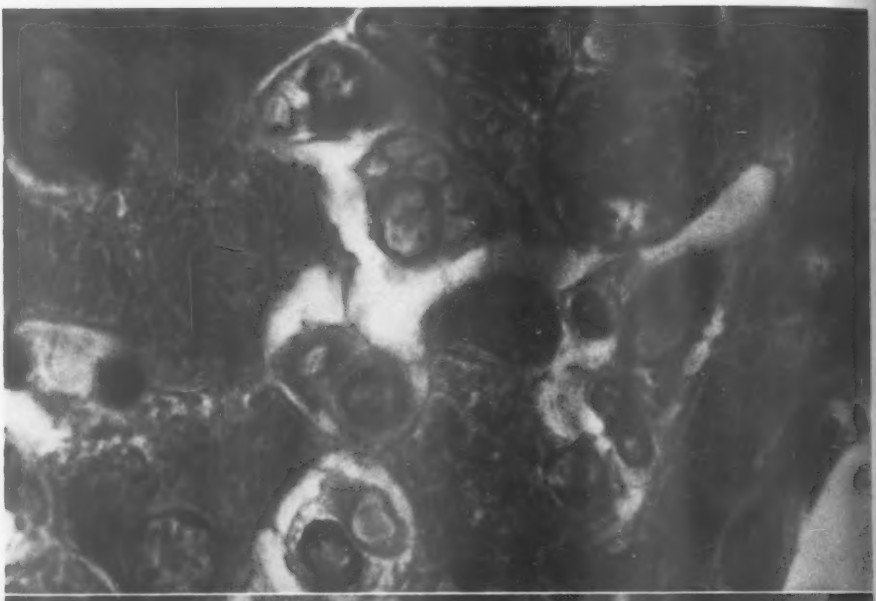

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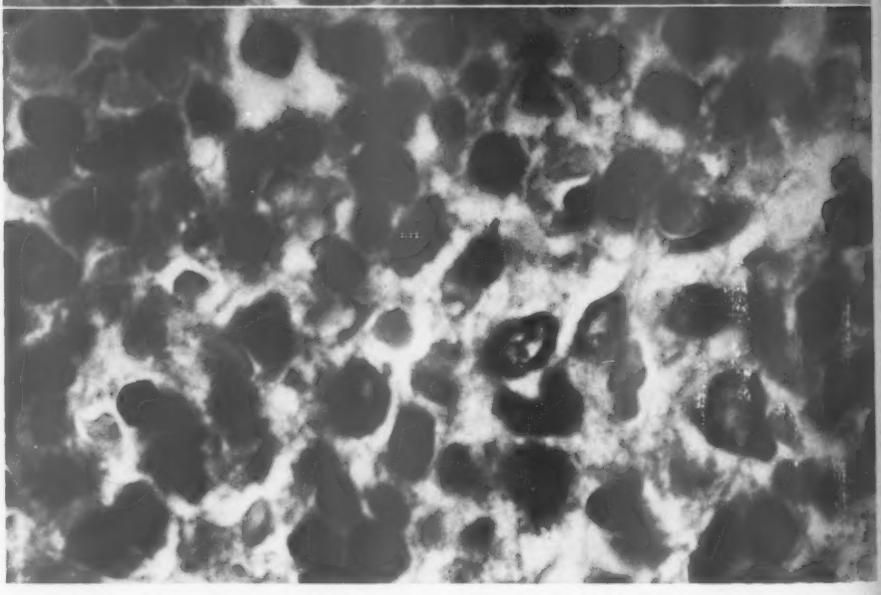

FIG. 6. Liver, mouse treated with zymosan (4 mg. over 8 days). Erythrophagocytosis is manifest in hyperplastic reticulum cells. × 1200.

FIG. 7. Spleen, mouse treated with zymosan (4 mg. over 8 days). Erythrophagocytosis by reticulum cells may be noted. × 1200.

RENAL HISTOCHEMISTRY OF OXIDATIVE ENZYME SYSTEMS IN AMINONUCLEOSIDE NEPHROSIS

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An experimental disease closely resembling human nephrosis can be produced in rats by the injection of an aminonucleoside related to puromycin, 6-dimethylamino purine, 3-amino-D-ribose. Frenk, Antonowicz, Craig and Metcoff [1] and Fiegelson, Drake and Recant [2] in their original descriptions of aminonucleoside nephrosis, mentioned that morphologic alterations in the kidney follow the onset of proteinuria. These changes were described as distention of convoluted tubules, fatty degeneration of tubular epithelium, cast formation and discrete thickening or fraying of glomerular basement membranes. Recent electron microscope studies of the renal lesion [3] pointed to mitochondrial swelling in the distal tubule cells and to alterations of the glomerular epithelium as early manifestations in aminonucleoside nephrosis. The relatively scanty evidence of morphologic renal alterations contrasts with the severe functional disturbances which occur in the course of the rapidly progressing experimental disorder, namely marked proteinuria, hypoproteinemia, hypercholesteremia, fluid retention and elevation of blood nonprotein nitrogen.

In the present paper, profound alterations of enzymatic activity in various portions of the nephron have been demonstrated in the course of aminonucleoside nephrosis. The functional alterations, as visualized by newer histochemical methods, were found to involve various mitochondrial enzyme systems and to be related to changes in mitochondria not detectable by conventional histologic techniques.

The observations recorded may provide a basis for the understanding of the functional aberrations which occur in acute nephrosis and may be helpful in further elucidating the mode of action of aminonucleoside at the cellular level.

Material and Methods

In the first experiment, 30 young male rats of an inbred albino strain (body weight 121 ± 3 gm.) were given daily subcutaneous injections of aminonucleoside (1.75 mg. per hundred gm. of body weight). The animals were fed commercial ("Nafag") pellets and had access to 1 per cent NaCl as drinking fluid. Previous experiments had shown that substitution of saline for tap water accentuated but did not qualitatively alter the histochemical changes in the kidney. Proteinuria was checked semiquantitatively by trichloroacetic acid precipitation. From the third day, 3 animals were sacrificed daily

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by cervical dislocation and were bled. The kidneys were removed immediately, weighed, and a slice from the midportion of the organ was quickly frozen in CO_2. The frozen tissue was stored at −20° C. and saved for histochemical examination; the rest of the renal tissue was fixed in formol-calcium and embedded in paraffin. Three untreated rats and 6 rats kept on saline for 13 days served as controls.

In a second experiment, designed to study the influence of increased fluid retention, the kidneys of 4 rats were examined 5 weeks after unilateral nephrectomy. These animals were kept on saline, received 3 subcutaneous injections of deoxycorticosterone acetate (50 mg. per kg. of body weight) as a microcrystalline suspension at weekly intervals, and were given 5 daily doses of aminonucleoside prior to sacrifice.

The possible recovery of aminonucleoside-induced renal lesions (experiment 3) was investigated in 10 animals which had received 9 daily injections of the drug. The kidneys were examined 7, 13, 25 and 43 days after the injections were discontinued. During this experiment, the rats were kept on tap water.

Histochemical Methods

For the demonstration of the activity of pyridine nucleotide-linked dehydrogenases and diaphorases in 5 μ cryostat sections, the methods of Hess, Scarpelli and Pearse[4,5] were used. Succinic dehydrogenase activity was demonstrated by the modified Pearse[6] method. Cytochrome oxidase activity was visualized by the modified Nadi reaction described by Nachlas, Crawford, Goldstein and Seligman.[7] Incubation was carried out at 37° C. for 15 minutes in order to demonstrate succinic dehydrogenase, cytochrome oxidase and diphosphopyridine nucleotide (DPN) diaphorase activity; for 30 minutes to demonstrate the activity of triphosphopyridine nucleotide (TPN) diaphorase and pyridine nucleotide-linked dehydrogenases.

Fresh-frozen sections were postfixed in formol-calcium and stained with toluidine blue and for lipids with oil red O. Five μ sections of paraffin-embedded tissue were stained with hemalum-eosin and the periodic acid-Schiff (PAS) procedure.

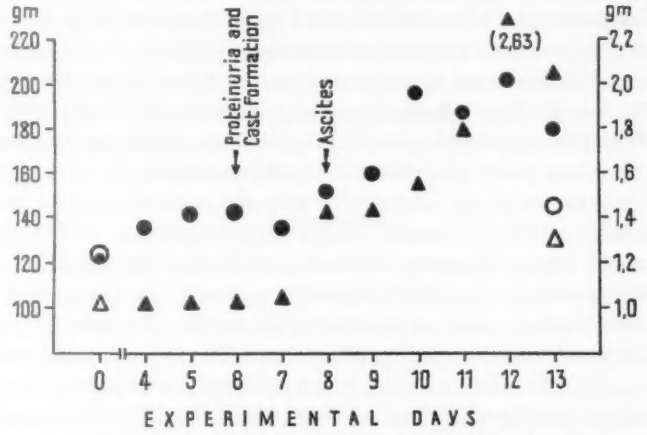
Results

The distribution in normal rat kidney of the various oxidative enzymes studied is referred to in previous publications.[4,5] The formazan pigment formed by the dehydrogenase reactions used and the indonaphthol purple produced by the cytochrome oxidase reaction appear as dots measuring 0.2 to 0.3 μ in diameter, situated at regular intervals on the mitochondrial membrane. After relatively brief incubation, the mitochondria remain small, provided that the reactions are performed in a hypertonic medium.[8] Any change in the size of the dye deposits after incubation of the sections in the usual manner can therefore be expected to reflect *in vivo* swelling of the mitochondria and thus allow for an assessment of mitochondrial structural alteration.[6] A normal mitochondrial pattern is depicted in Figure 12.

Experiment 1

The influence of aminonucleoside treatment on body and kidney weights are presented diagrammatically in Text-figure 1. Proteinuria had developed in all the experimental animals by the sixth day; subcutaneous edema and ascites were present by the eighth day. The enzymatic alterations in the kidneys can be described as follows:

Fourth to Sixth Days. The earliest alteration at 4 days consisted of discrete mitochondrial swelling in cells irregularly distributed in the middle and distal parts of the proximal convolutions (succinic dehydrogenase and DPN-diaphorase reaction; Fig. 1). By the sixth day the swelling became more marked, involved cells of the proximal parts of



TEXT-FIGURE 1. Body weight (●, left hand scale) and weight of both kidneys (▲, right hand scale) of nephrotic rats. Open signs indicate control animals. Each sign represents the mean of 3 rats.

the first convoluted tubules, and appeared also in more distal parts of the distal convolutions (Fig. 2). Concomitant with the first appearance of PAS-reactive protein casts, there was distention of proximal tubules. The enzymatic pattern was unaltered, except for a definite increase of TPN-dependent isocitric dehydrogenase activity in collecting ducts throughout the medulla. The swollen mitochondria were almost regularly situated in the basal parts of the tubular cells. At 6 days, more marked mitochondrial swelling was accompanied by the appearance of small lipid droplets in the cytoplasm close to the basement membrane (Fig. 3). PAS-staining droplets were seen in the perinuclear region of proximal tubule cells, obscuring the brush border structure.

Seventh to Eighth Days. Mitochondrial swelling, visualized by all the dehydrogenase reactions used, was very prominent in every part of the proximal tubules (Fig. 6) and was accompanied by tubular dilatation. Distended tubules often contained desquamated tubular cells which were still enzymatically active (Fig. 5). More advanced mitochondrial swelling resulted in strongly active organelles which finally coalesced. Lipid droplets, the appearance of which seemed to be closely related to advanced mitochondrial swelling, displayed a residual enzymatic activity in

the form of a peripheral formazan deposit (Fig. 4). Slight mitochondrial alterations were noted in both the descending and ascending limbs of Henle's loop. No further change was noted in the collecting ducts. The activity of succinic, DPN-isocitrate and malate dehydrogenase was diminished in the proximal tubular epithelium. β-Hydroxybutyrate dehydrogenase activity became very low in the ducts of the outer medulla. TPN-dependent activity was preserved throughout the nephron.

At 8 days, the activity of cells composing the glomerular tufts became conspicuously increased. This increase involved DPN-dependent activity (Fig. 6), namely lactic, glutamic and α-glycerophosphate dehydrogenase, as well as TPN-linked enzymes. The appearance of glucose-6-phosphate dehydrogenase activity in glomerular cells was especially striking, since these cells normally are nonreactive with the cobalt-formazan method. Glomerular activity increased further with progression of the tubular lesions and was paralleled by thickening of the capillary basement membranes due to deposition of PAS-positive material. No evidence of mitochondrial swelling could be detected in glomerular elements.

Cytochrome oxidase activity was preserved even in extremely swollen mitochondria. In cells containing much lipid, intracellular localization of this enzyme was partially lost because of lipid-solubility of the reaction product (indonaphthol purple; Fig. 7).

Dilatation of proximal and distal ducts became marked with progression of the disorder, and mucoprotein casts were seen in these ducts as well as in collecting ducts of the outer medulla. Interstitial edema of renal tissue was evident histologically in cryostat sections and was also reflected in the progressively increasing kidney weight.

Ninth to 13th Days. Further development of the lesions in the proximal tubules was apparent by the loss of tricarboxylic acid cycle activity in this segment. This began in the distal parts and progressed toward the glomerulus (Fig. 8). DPN- and TPN-diaphorase activity and cytochrome oxidase activity were partially preserved and bound to grossly swollen mitochondria. The enzymatic activity of the distal tubules was only a little impaired, in spite of marked dilatation of all the cortical tubules. Glucose-6-phosphate dehydrogenase activity of the macula densa of the distal tubule was preserved until the terminal stages (Fig. 9). The activity of this enzyme, together with TPN-diaphorase and DPN-dependent enzymes became increasingly strong in the glomerulus (Figs. 10 and 11) and also in the muscle cells of the arteries. The collecting ducts of the papillas showed increased activity of glutamic, lactic, ethanol, α-glycerophosphate and TPN-isocitric dehydrogenase. It may be significant that this enzyme activation was not followed by a similar increase in cytochrome oxidase activity which is normally very low in the

papillary ducts. The activity of the intercalated dark cells of the cortical collecting ducts was not visibly altered. The ascending and descending limbs suffered entire loss of succinic and β-hydroxybutyrate activity, although the mitochondrial alterations were less pronounced than in the proximal tubules.

Concomitant with the enzymatic changes, marked interstitial edema of kidneys was accompanied by both dilatation of ducts and cast formation. This was especially prominent in inactive segments of proximal tubules. At 12 days, whole cortical segments were entirely devoid of enzymatic activity, although apart from cloudy swelling and fatty degeneration, the tubular epithelium had retained its morphologic integrity, as shown in conventionally stained histologic sections.

Experiment 2

Unilateral nephrectomy, deoxycorticosterone administration and salt load prior to the injection of aminonucleoside led to severe alterations in the proximal tubules. Five daily doses of the drug caused widespread focal necrosis of proximal tubular cells, gross mitochondrial swelling and loss of enzymatic activity.

The distal tubules, however, suffered only little alteration. As in experiment 1, the glomerular cells displayed high dehydrogenase activity and contained apparently intact mitochondria.

In the intact or unilaterally nephrectomized animal, deoxycorticosterone and salt load alone, within about 2 weeks, led to mitochondrial swelling and increased activity of succinic dehydrogenase and DPN-diaphorase. In the distal tubule, a gradual reduction of glucose-6-phosphate dehydrogenase activity occurred without evident mitochondrial alteration. These observations have been recorded in a previous communication.[9]

Experiment 3

Seven days after aminonucleoside administration was discontinued, no restitution of the mitochondrial and glomerular alteration was apparent. The lesions corresponded to the change seen at the time of aminonucleoside withdrawal (8 days), or had even progressed in scattered segments of the proximal tubules. At 13 days, these segments had lost most of their enzymatic activity, whereas the mitochondrial configuration and activity had returned to normal in those proximal segments which had had less alteration initially. Twenty-five and 43 days after the administration of aminonucleoside, scattered proximal tubules were found to have undergone complete destruction and scarring. Others, still dilated, were covered with regenerating epithelium exhibiting weak

DPN- and TPN-diaphorase activity. Spurious mitochondrial swelling could still be detected in preserved proximal tubules. The glomerular cells frequently showed mitochondrial swelling and had retained their increased dehydrogenase activity. The basement membranes of the glomerular tufts were broadened, and large PAS-staining droplets were noted in the glomerular epithelium.

Discussion

The sequence of renal alterations observed in aminonucleoside nephrosis in rats was shown histochemically to begin with mitochondrial alterations in the epithelium of the proximal tubules. These changes, involving the activity of various oxidative enzymes, arose before the appearance of both marked proteinuria and tubular distention. Early mitochondrial swelling was always observed to occur in the basal portions of the cells. This contrasted with swelling following protein absorption, which takes place in the portion facing the lumen.[10] With the development of nephrosis, mitochondrial swelling rapidly progressed and was accompanied by marked reduction in the activity of tricarboxylic acid cycle enzymes and early loss of β-hydroxybutyrate dehydrogenase activity. With the onset of edema and the appearance of ascites, the mitochondrial lesions of the proximal tubules were fully developed. The mitochondrial activity of distal tubular cells showed only minor changes.

Translated into enzymatic processes *in vivo*, the histochemical features of marked mitochondrial alteration are suggestive of profound disturbances of energy-yielding mechanisms within tubular cells. It is remarkable that damage to the energy-supplying citric acid cycle enzymes clearly preceded alterations in the activity of dehydrogenases linked with glycolysis or protein metabolism.

Mitochondrial swelling is indicative of uncoupling of oxidative phosphorylation in the cells affected.[11] This implies that the antimetabolic action of aminonucleoside interferes somehow with cellular reactions linked to the production of high energy compounds. In a crude yeast system, it has been demonstrated by Kessner, Borowsky and Recant[12] that aminonucleoside exerts an inhibitory effect on ATP production from adenosine in the presence of inorganic phosphate, probably by inhibiting the adenosine phosphokinase reaction. Bartlett and Shelata[13] demonstrated that, following administration of tritiated aminonucleoside to rats, a significant portion of the activity found in the acid soluble nucleotide fraction in kidney and liver tissue was due to labeled cytidin diphosphate and adenosine monophosphate. Aminonucleoside did not interfere with synthesis of pyridine nucleotide coenzymes, such as DPN.

Since mitochondrial alterations in the proximal tubular epithelium

resulting from deoxycorticosterone and salt overdosage[9] were similar to those found after aminonucleoside administration, a possible action of mineralocorticoids on the tubular epithelium must be considered in the latter condition. An enhancing effect by corticoid hormones on the tubular alterations in aminonucleoside nephrosis has been demonstrated in experiment 2. Increased excretion of aldosterone in the adrenal vein blood has been found[14] to precede edema in nephrotic rats. Later reports provided evidence that hyperaldosteronism was responsible for the severe fluid retention in aminonucleoside nephrosis.[15,16] Since fluid retention has been observed in adrenalectomized nephrotic rats maintained on both saline and a minimum dose of desoxycorticosterone,[17] it seems likely that increased aldosterone secretion is not the only cause of fluid retention. It may well be the consequence of intravascular fluid loss accompanying nephrotic edema. In aminonucleoside nephrosis, in contrast to deoxycorticosterone-salt overdosage, there is a reduction of neither macula densa activity nor the renin content of kidney tissue.[17] This suggests further that the action of the antimetabolite is not on corticoid production or excretion primarily.

The marked reduction of renal β-hydroxybutyrate dehydrogenase activity may result from direct interference of aminonucleoside with mitochondrial fatty acid oxidation, possibly by blocking the ATP-dependent formation of fatty acid adenylate. This enzymatic step is involved in the formation of the coenzyme A derivative.[18]

The increased activity of glutamic dehydrogenase and of several enzymes linked with glycolysis in the collecting ducts may be the result of activation by mitochondrial damage or may represent true enzymatic adaptation. The high activity of TPN-diaphorase in the same area, and the preserved activity of DPN-dependent systems (with the exception of DPN-isocitrate and malate dehydrogenase), may signify increased hydrogen transfer via the transhydrogenase reaction. Throughout the kidney, cytochrome oxidase activity was altered only as a result of mitochondrial swelling and seemed not to be influenced by aminonucleoside directly.

Increased glomerular activity was observed to parallel the progression of tubular lesions and to accompany proteinuria. This is suggestive of an important metabolic adaptation of glomerular cells to altered tubular reabsorptive capacity, probably intended to maintain "glomerular-tubular balance."[19] An increased activity of both diaphorase and of the glutamic, lactic, ethanol and α-glycerophosphate dehydrogenases was found to occur without any detectable mitochondrial swelling and, therefore, is most likely due to an increase in the amount of enzyme protein. In view of the possible role of glucose-6-phosphate dehydrogenase in

biosynthetic processes,[20] the marked increase in activity of this enzyme in glomerular cells seems to be significant. The histochemical findings of marked alteration in the pattern of glomerular activity fully support recent observations made by quantitative microchemical methods on glomerular enzymatic changes in nephrotic animals.[21]

The meaning of altered enzymic activity in the glomerulus may further be elucidated by extending microchemical and histochemical studies both to various types of experimental nephrosis and nephritis and to the analysis of human biopsy specimens. Such enzymatic alterations are by no means specific. Activation of glomerular glucose-6-phosphate dehydrogenase has been observed to follow sustained renal ischemia in animals bearing a Goldblatt clamp.[22] In a manner obscure at present, the enzymatic glomerular changes in nephrosis are possibly related to thickening of the basement membranes, to swelling and coalescence of epithelial foot processes and to increase in the number of epithelial cytoplasmic vacuoles.[3] In contradistinction to ultrastructural lesions reported to be reversible,[3] enzymic glomerular activity was still found to be altered 43 days after 9 daily injections of aminonucleoside. By that time, most of the tubular activity had been restored.

The observations made in this study point strongly to the mitochondrial metabolism in the proximal tubular system as the primary target of the antimetabolic activity of aminonucleoside. The glomerular alterations observed seemed to be of secondary order although a direct action of the "fraudulent" nucleoside on glomerular structure or permeability could not be excluded.

It seems possible that proteinuria in aminonucleoside nephrosis represents, to a considerable extent, failure of tubular absorption. Tubular failure also might explain the high rate of albumin excretion[2,23] since plasma albumin (which normally is present in the glomerular filtrate) is absorbed by intact proximal tubule cells.[24]

Summary

The renal alterations induced by daily injections of 6-dimethylaminopurine, 3-amino-D-ribose (aminonucleoside) were investigated histochemically in young rats. With the use of methods for the precise intracellular localization of various oxidative enzymes, the first change was found to occur in segments of the proximal tubules and to consist of mitochondrial swelling in basal parts of the affected cells. With the development of nephrosis, mitochondrial swelling involved the entire proximal convolution and was followed by progressive reduction in the activity of tricarboxylic acid enzymes and β-hydroxybutyrate dehydrogenase. The distal tubules showed only little alteration of mitochondrial activity. An

increased activity of TPN-dependent enzyme systems was found in the collecting ducts.

After 8 days of aminonucleoside administration, a progressively increasing activity of glucose-6-phosphate dehydrogenase and of dehydrogenases linked to glycolysis was observed in glomerular cells. This alteration occurred without demonstrable mitochondrial swelling and was accompanied by thickening of the glomerular basement membrane. In rats recovering from 9 injections of aminonucleoside, the enzymatic glomerular alterations persisted for at least 43 days, whereas the tubular changes showed partial reversal.

Sodium retention induced by salt load and deoxycorticosterone overdosage before the administration of aminonucleoside, greatly accelerated damage to the proximal tubular system in unilaterally nephrectomized rats and led to epithelial necrosis.

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[*Illustrations follow*]

Legends for Figures

Fig. 1. Slight mitochondrial swelling in proximal tubular cells after 4 days' treatment with aminonucleoside. DPN-diaphorase reaction. × 1000.

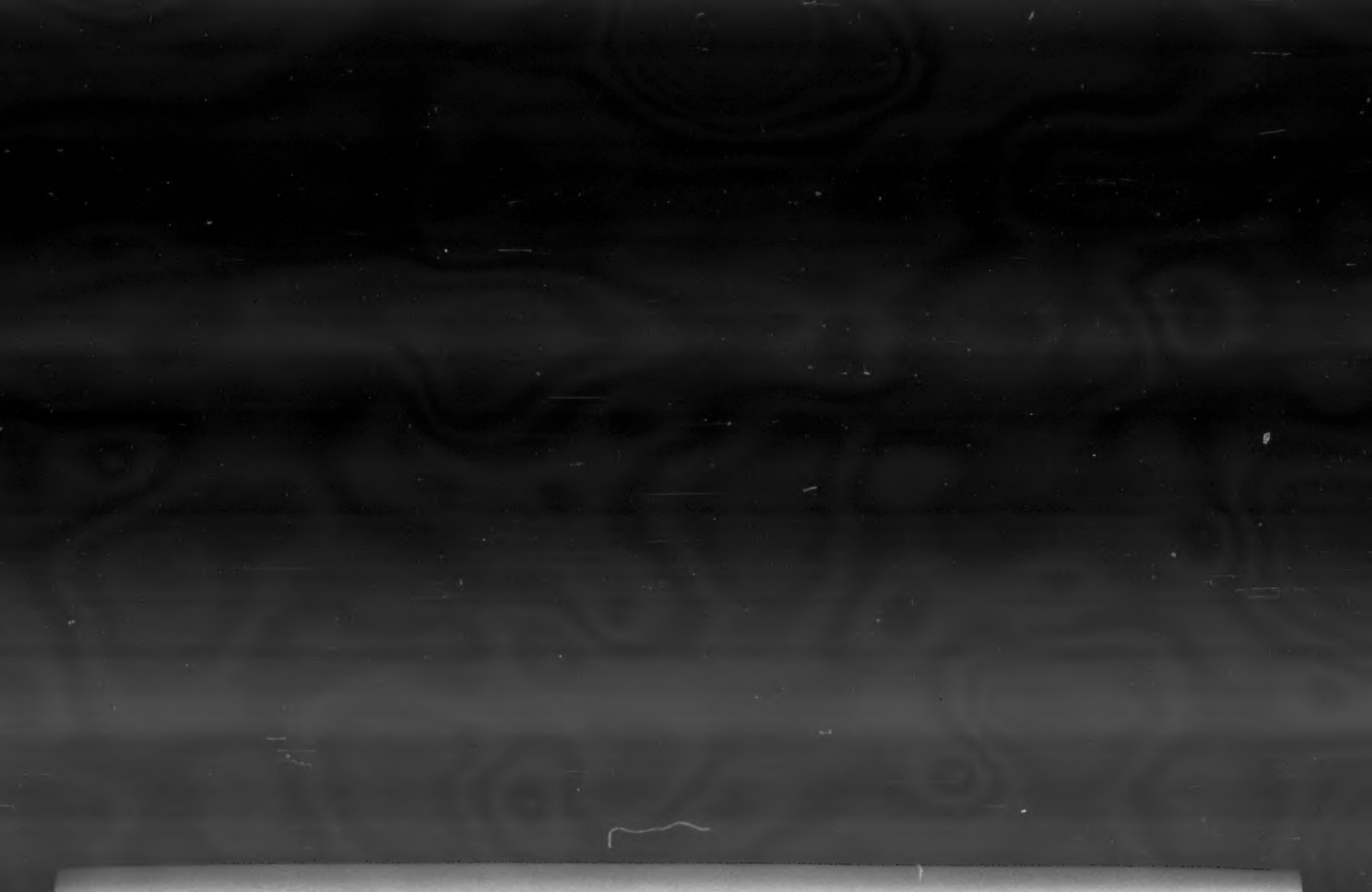
Fig. 2. At 6 days, mitochondrial swelling is more pronounced in segments of proximal tubules and is mainly restricted to basal portions of the cells. Succinic dehydrogenase reaction. × 1000.

Fig. 3. Parallel section to Figure 2. Lipid droplets appear at sites of marked mitochondrial swelling. Oil red O stain. × 1000.

Fig. 4. After treatment with aminonucleoside for 8 days, mitochondrial swelling is progressive in proximal tubular cells. Reactive sites are shown to coalesce and often to adhere to faintly stained lipid droplets. Succinic dehydrogenase reaction. × 1000.

Fig. 5. Same kidney shown in Figure 4. Accumulation of enzymatically active desquamated epithelium in distended portions of proximal tubules. Mitochondrial swelling is evident. DPN-diaphorase reaction. × 450.

Fig. 6. After treatment for 8 days, marked mitochondrial swelling involves the whole of the proximal tubules. Glomerular cells show increased activity. DPN-diaphorase reaction. × 450.



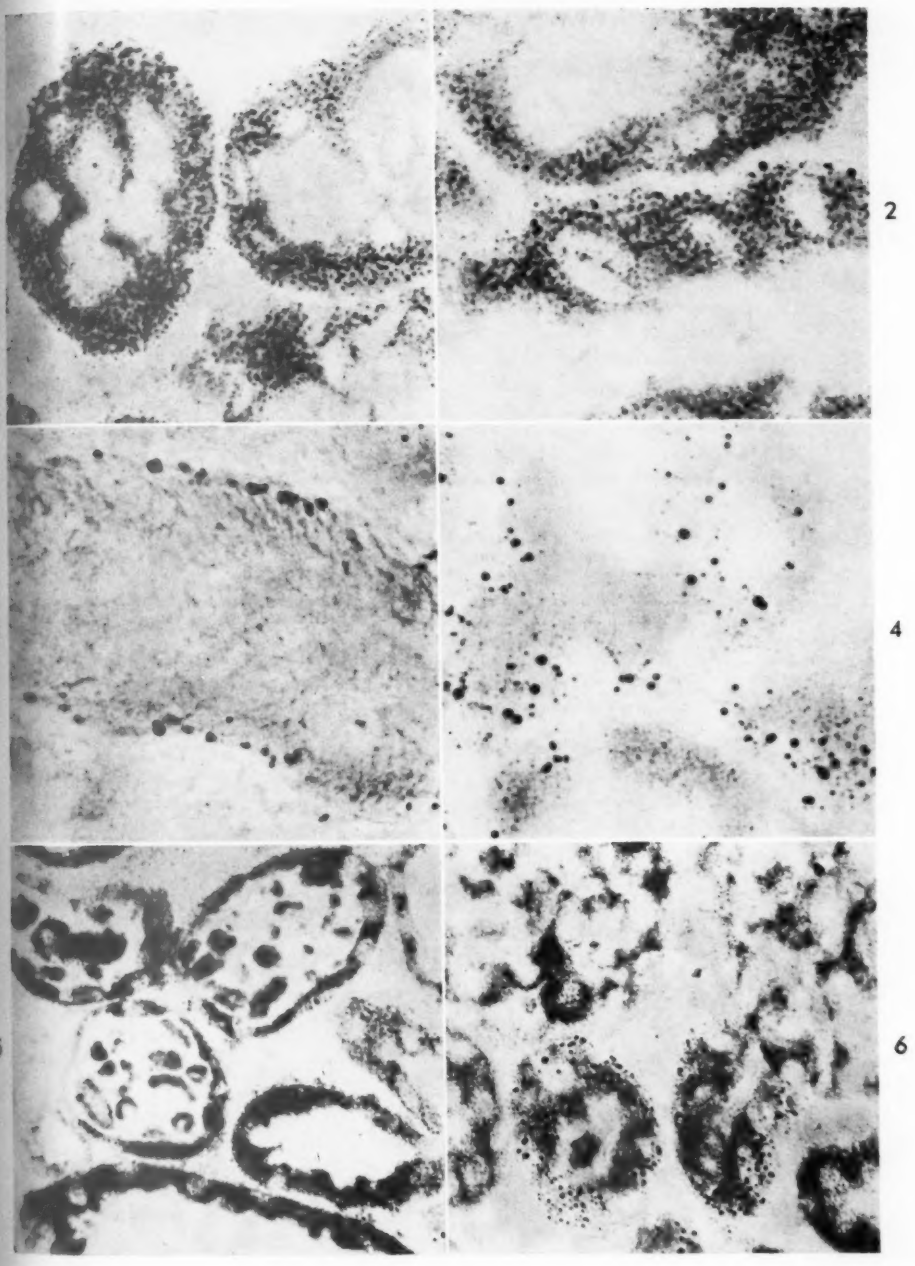

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FIG. 7. Cytochrome oxidase activity is preserved after 9 days of treatment with aminonucleoside. The enzymatically formed indonaphthol purple partially dissolves in lipid droplets of altered proximal tubular cells. A distal tubule (left side) shows little mitochondrial swelling. × 680.

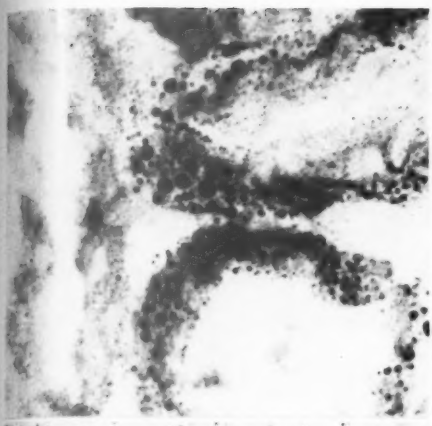
FIG. 8. Marked reduction of succinic dehydrogenase activity in portions of proximal convolutions. Twelve days' treatment. × 320.

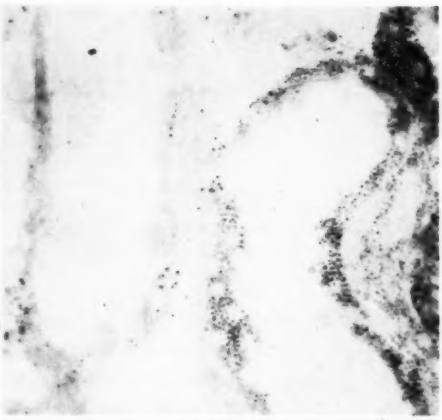
FIG. 9. At 9 days, a moderately strong (normal) activity of glucose-6-phosphate dehydrogenase is shown in the macula densa of a distal tubule. × 1000.

FIG. 10. Part of a glomerulus with increased activity of glucose-6-phosphate dehydrogenase in scattered glomerular cells. Nine days' treatment. × 1000.

FIG. 11. High activity of TPN-diaphorase in glomerular cells. The intact mitochondrial structure of the glomerulus contrasts with grossly swollen mitochondria in adjacent parts of the proximal tubules. Twelve days' treatment. × 450.

FIG. 12. Normal mitochondrial pattern in proximal tubule cells of a control animal. On the left side, an adjacent glomerulus contains weakly active cells (compare with Fig. 6). DPN-diaphorase reaction. × 1000.





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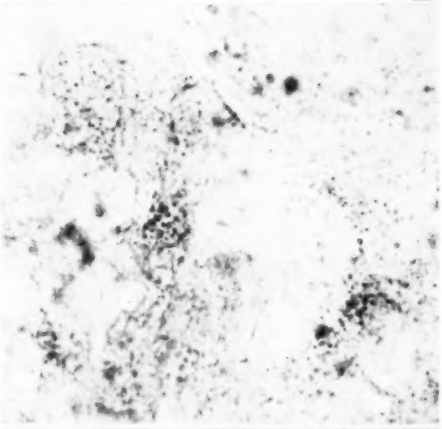

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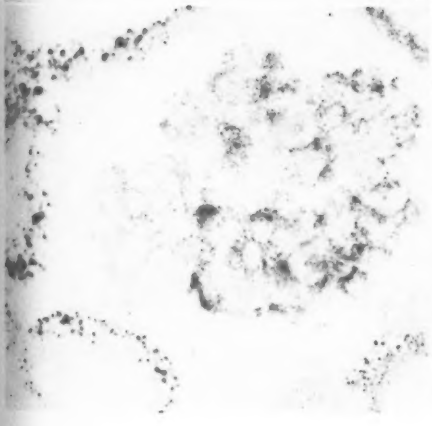

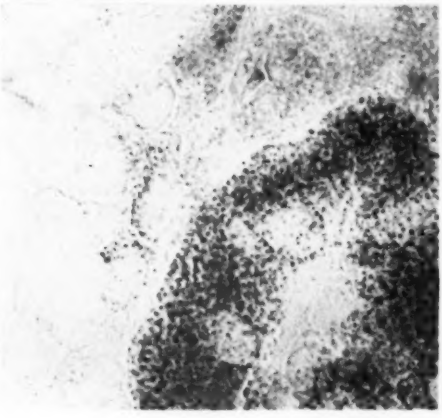

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HISTOCHEMICAL DEMONSTRATION OF SIALOMUCIN IN TRANSPLANTABLE THYROID CARCINOMA

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From recent histochemical studies of epithelial mucins, methods have evolved for distinguishing between certain sialomucins and sulfated acid mucopolysaccharides.[1,2] Biochemical assays have demonstrated the presence of sialic acid in tumors,[3] but sialomucins have not thus far been localized histologically or biochemically in neoplastic cells or their secretions.

Thyroid carcinomas arising in rats fed thiouracil in this laboratory have developed through transplantation into a number of lines of widely differing histologic appearance.[4] Homogenates of tumors of one of these lines are very viscous. Histologic sections of this and other variants show abundant intracellular or intrafollicular periodic acid-Schiff (PAS) stained substance suggesting the presence of mucin. It appeared of interest to apply methods for localizing and characterizing sialomucins to the investigation of the secretions of these thyroid carcinomas. The investigation has shown that some of the tumors secrete neutral mucopolysaccharide, histochemically comparable to that of normal thyroid colloid, whereas others contain or secrete acid mucopolysaccharide, the basophilia of which is accounted for entirely by enzymatically removable N-acetylneuraminic acid.

Experimental Methods

The transplanted thyroid tumors studied were carried subcutaneously in male Fischer rats. They were identified as thyroid tumors on the basis of origin, structure and function (radioiodine metabolism). In addition, some undifferentiated or anaplastic lines were considered to be of thyroid origin because they arose as variants in the implantation sites of tumors with mixed histologic pattern, part of which was clearly thyroid in character.

Tumor tissues obtained immediately post mortem from animals carrying the various lines of thyroid tumors were fixed 24 hours in neutral formalin prior to paraffin embedding and sectioning at a thickness of 5 μ. Sections were stained 30 minutes in 0.1 per cent Alcian blue in 3 per cent acetic acid followed by a PAS stain (AB-PAS) according to the combination of these methods described by Mowry.[5] This method stains most acid mucins blue or purple and neutral mucins red. Alternatively, sections

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were stained 30 minutes in 0.02 per cent azure A in phosphate-citrate buffer at pH 1.5, 2.5 and 4.[1] After the staining with azure A, the sections were dehydrated through graded alcohols or preferably were blotted dry before mounting with cellulose caprate. Other sections were stained 5 hours in 0.1 per cent N,N-dimethyl-*p*-phenylenediamine after periodic acid oxidation, as a means of localizing neutral mucopolysaccharides according to the periodic acid diamine (PAD) procedure recently developed and described elsewhere.[6] Finally a 1-hour aldehyde blockade with 5 per cent aqueous phenylhydrazine hydrochloride was interposed between periodic acid and Schiff reagent in a periodic acid-phenylhydrazine-Schiff (PAPS) variant of the conventional PAS stain as a means of selectively coloring acid mucins (Spicer, S.S., unpublished method).

The "mild methylation" modification of the Fisher-Lillie methylation blockade of tissue basophilia involved a 4-hour exposure of tissue sections at 37° C. to methanol containing 0.1 N HCl; removal of methyl esters thus induced was accomplished by 20 minute saponification of the collodionized slides in 70 per cent ethanol containing 1 per cent KOH as previously described.[1]

Sialidase was purified 25-fold from culture filtrates of *Clostridium perfringens*, according to the unpublished method of R. E. Feeney, M. B. Rhodes, and J. S. Anderson (Department of Biochemistry and Nutrition, University of Nebraska) and 1,500-fold from a culture of *Vibrio cholerae* by the method of Ada and French.[7]

To determine the effect of sialidase on tissues, deparaffinized, air-dried, histologic sections were layered with about 0.1 ml. of enzyme buffer solution containing 5 γ of protein in the case of the *Vibrio cholerae* and 35 γ in the case of the *Clostridium perfringens* preparation. This solution (pH 5.25) contained 0.1 M citrate and 0.04 M calcium ions. These and adjacent control sections, flooded with the buffer solution alone, were incubated 8 to 16 hours at 39° C. The slides were supported on glass rods close above a layer of water in covered Petri dishes. After digestion, the supernatant fluid was aspirated with a fine pipette. This fluid was combined with two 0.1 ml. rinse volumes and assayed for sialic acid by the thiobarbituric acid method.[8] Some of the sections were stained; duplicates were scraped into a test tube, hydrolyzed in 0.2 ml. of 0.1 N H_2SO_4 at 80° C. for 1 hour to release the bound sialic acid and assayed by the thiobarbituric acid method. The total sialic acid concentration in fresh tissue and deparaffinized embedded tumor tissue was determined by this method after homogenizing and then hydrolyzing similarly. The chemical form of the sialic acid in the neoplasm was determined by chromatography of the acid-hydrolyzed tissue homogenate. The sialic acid was concentrated from the hydrolysate by absorption on Norit A and elution with 60 per cent ethanol. It was chromatographed by the ascending method for 24 hours on Whatman No. 1 paper in two solvents capable of separating N-glycolyl from N-acetylneuraminic acid. Solvent 1 was N-butanol:N-propanol:0.1 N HCl, 1:2:1[9]; solvent 2 was ethanol:water:ammonia, 80:20:1. Sialic acids were located on the chromatogram by the thiobarbituric acid spray reagent.[10]

Results

Histochemical Characteristics of Normal Thyroid Colloid

Normal thyroid colloid stains red or, perhaps more accurately, magenta with the Alcian blue-PAS method, indicating the presence of neutral mucopolysaccharide (Fig. 1). The failure to stain with azure A at pH 2.5 (Fig. 2) demonstrated the absence of strongly dissociated free carboxylic groups and sulfate esters. The orthochromatic blue coloration by this dye at pH 4 (Fig. 3) was consistent with the presence of weakly dissociated acidic groups such as the carboxyls of protein.

Alcian Blue-PAS Staining of Rat Thyroid Carcinomas

Two types of mucinous secretion were observed in the rat thyroid carcinomas. In one type the follicular fluid (colloid) resembled that of normal thyroid in its red coloration with the Alcian-blue PAS stain (Fig. 4). The follicular tumors secreting such neutral mucopolysaccharide, as a rule, had well formed, only moderately irregular, rather uniformly small follicles. A nonfollicular variant with a neutral mucinous secretion (tumor line 16-1) formed, instead of follicles, medullary sheets or lobules of cells, many of which contained a globule of PAS-positive, nonbasophilic, nondiastase-digestible substance.

In the other type of secretion, the follicular fluid when stained with the Alcian blue-PAS method varied from red observed in a few follicles and purple seen occasionally, to dark blue staining present in the majority of the follicles (Fig. 5). Such purple to blue staining pointed to the existence of mucin-containing acid groups such as carboxyls or sulfate esters. The follicular tumors secreting acid mucopolysaccharide, in general, showed irregular follicles of varied size and occasional pools of mucus apparently occupying stromal septums, unenclosed by epithelial cells. A nonfollicular variant with an acid mucous secretion formed medullary sheets of cells (Fig. 9). The Alcian blue-PAS stain visualized histochemically variable mucopolysaccharide distending these cells. The abundant cytoplasm was filled in most cells with purple or blue, but in a few, with red staining mucin.

Azure A Staining of Tumor Mucins

The neoplastic follicles showing neutral mucopolysaccharide with the Alcian blue-PAS stain resembled follicles in normal thyroid also in their lack of basophilia toward azure A at pH 2.5 and their orthochromatic blue staining at pH 4. In the more bizarre follicles showing acid mucopolysaccharide with the Alcian blue-PAS method, purple metachromasia was observed with azure A at pH 2.5 (Fig. 7). The acid mucopolysaccharide in the nonfollicular variant also colored metachromatically at pH 2.5 with azure A (Fig. 11). The follicular fluid and epithelial cytoplasm in tumors failed to stain with azure A below pH 2. Metachromasia present at pH 2.5 and absent at pH 1.5 could be due to sulfate esters since the normal strong dissociation of these groups may be partially masked by protein, according to the mechanism suggested by French and Benditt.[11] Such staining may, on the other hand, reflect the presence of strongly dissociated carboxyls such as those in sialomucins.[12,13]

TABLE 1

HISTOCHEMICAL CHARACTERISTICS OF MUCOPOLYSACCHARIDE IN SECRETIONS OF THE NORMAL AND NEOPLASTIC RAT THYROID

		Alcian blue-PAS			Azure A, pH 2.5		PAD *			PAPS †	
	Tumor lines	Untreated section	After mild methylation	Mild methylation + KOH ‡	Untreated section	After mild methylation	Untreated section	After mild methylation	Aldehyde fuchsin-Alcian blue	Untreated section	After mild methylation
Normal thyroid colloid		++++ Red	++++ Red	++++ Red	Unstained	Unstained	+++ Orange-brown	+++ Orange-brown	± Purple	Unstained	Unstained
Follicular colloid, carcinomas with neutral MPS §	1-2 and 1-1C	++++ Red	++++ Red	++++ Red	Unstained	Unstained	+++ Orange-brown	+++ Orange-brown	± Purple	Unstained	Unstained
Follicular colloid, carcinomas with acid MPS	1-4 and 1-8	++++ Purple-blue	++++ Red	++++ Purple-blue	++++ Purple	Unstained	Unstained	++ Orange-brown	+++ Blue	+++ Red	Unstained
Intracellular acid MPS, tumor cells	16-3 16-4 16-5	++++ Purple-blue	++++ Red	++++ Purple-blue	+++ Purple	Unstained	Unstained	++ Orange-brown	+++ Blue	+++ Red	Unstained

* Periodic acid-diamine.
† Periodic acid-phenylhydrazine-Schiff.
‡ De-esterification by saponification interposed between methylation and staining.
§ Mucopolysaccharide.

TABLE II

EFFECT OF SIALIDASE DIGESTION ON THYROID MUCINS

	Buffer control					Enzyme digested				
				Sialic acid					Sialic acid	
	Alcian blue-PAS	Azure A pH 2.5	PAD †	In tissue section (μg.)	In supernatant fluid (μg.)	Alcian blue-PAS	Azure A pH 2.5	PAD †	In tissue section (μg.)	In supernatant fluid (μg.)
Follicular thyroid carcinoma with acid MPS *	++++ Purple-blue	++++ Purple	Unstained	2.7	0	++++ Red	Unstained	+++ Orange-brown	0.4	2.8
Thyroid carcinoma with intracellular acid MPS	++++ Purple-blue	+++ Purple	Unstained	2.1	0	++++ Red	Unstained	+++ Orange-brown	0.3	2.4
Follicular thyroid carcinoma with neutral MPS	++++ Red	Unstained	+++ Orange-brown			Unaltered	Unaltered	Unaltered		
Normal thyroid colloid	++++ Red	Unstained	+++ Orange-brown	3.1 ‡	0.2 ‡	Unaltered	Unaltered	Unaltered	0.5 ‡	2.3 ‡

* Mucopolysaccharide.

† Periodic acid-diamine stain.

‡ Section contained several glands embedded in one block.

Additional Histochemical Characteristics of the Tumor Acid Mucopolysaccharide

Experience with the 6-hour periodic acid-diamine (PAD) stain has demonstrated its selectivity for neutral mucopolysaccharides.[6] This method failed to color the acid mucins in the rat thyroid carcinomas, but imparted a typical orange-brown staining in the neutral mucins in these tumors as it did in normal thyroid colloid (Table I). The periodic acid-phenylhydrazine-Schiff (PAPS) procedure, on the other hand, stained the acid but not the neutral mucins in tumors and normal thyroid colloid (Table I).

As a further means of identifying the acid group in secretions of rat thyroid cancers, blockade of basophilia by a mild methylation was investigated. This procedure has been found to block selectively the acidic staining characteristics of nonsulfated acid mucins.[1] Mild methylation eliminated the basophilia in the secretions of the rat thyroid cancers (Table I). Saponification applied after the methylation restored Alcian blue reactivity to these mucins. After mild methylation the acid mucins in the neoplasms no longer stained by the PAPS method, but became PAD-reactive, giving an orange-brown color with this reagent in 6 hours. Like known nonsulfated acid mucins, those in the thyroid carcinomas stained with Alcian blue in an aldehyde fuchsin-Alcian blue sequence and stained weakly with aldehyde fuchsin in the reverse procedure. Sulfated mucins usually color purple with the aldehyde fuchsin in the first sequence and often stain blue in the latter.[14]

Characterization of the Acid Moiety in the Rat Thyroid Carcinomas

Specific identification of the acid material in the follicular fluid or the neoplastic cells as sialomucin stemmed from removal of the basophilia by digestion with purified sialidase from two different sources (Figs. 6, 8, 10 and 12; and Table II). Specific sialic acid assay[8] of the digested and undigested sections as well as the supernatant fluid following digestion further established the identity of the acid group in these secretions (Table II). Digestion released essentially all of the sialic acid from the sections into the supernatant fluid. At the same time it removed Alcian blue affinity and metachromasia with azure A. It was apparent that the acid group was hydrolyzed without further degradation of the polysaccharide. Thus the PAS reactivity remained unimpaired after digestion, and the Alcian blue-PAS method gave the red coloration of neutral mucin. Moreover, after sialidase digestion the PAD method imparted the orange-brown color of neutral mucopolysaccharide to the tumor mucins. Although treatment with sialidase removed the meta-

TABLE III

COMPARISON OF TYPE OF MUCIN, SIALIC ACID CONCENTRATION, STRUCTURE, AND FUNCTIONAL ACTIVITY IN THYROID CARCINOMA

Tumor line	Generation	Type of mucin	Sialic acid concentration		Structure *	Function *	
			μM/gm. dry wt.	μM/gm. wet wt.		Iodide concentration	Protein-bound I^{131} formation
1-1A	22, 24	None	6.7		Cellular	+	−
1-1B	27	Neutral			Cellular, infrequent small follicles	−	−
1-1C	24, 26	Neutral			Small to medium follicles with colloid	+	+
1-1D	19	Neutral		1.7	Cellular, infrequent tiny follicles with colloid	+	−
1-2	17, 18	Neutral	10.3		Follicles with colloid	+	+
1-3	32	None	13.0		Cellular	−	−
1-4	16, 17	Mostly acid	35.2	6.4	Irregular follicles with colloid	−	−
1-5A	25, 27	None	11.4	1.0	Small follicles, no colloid	+	−
1-5B	28	Neutral			Cellular, very infrequent small follicles with colloid	−	−
1-5C	21	Neutral			Cellular, infrequent follicles with colloid	±	+
1-5D	22	Neutral			Cellular, occasional follicles with colloid	+	−
1-5E	25	Neutral			Small follicles with colloid	−	−
1-5F	18, 20	Neutral and acid	26.9		Cellular, follicles with colloid	±	+
1-5G	24	Neutral			Cellular, occasional small follicles with colloid	+	−
1-6	24	None			Cellular	−	−
1-7	22, 23, 25	None	11.1	2.2	Flat, empty follicles	+	−
1-8	20, 21, 22	Neutral and acid	25.9	4.9	Follicles with colloid	+	+
1-9	18	Neutral and acid	29.6		Cellular, small follicles with colloid		
1-9	19, 20	None		2.6	Cellular, small follicles without colloid	+	−
16-1	34	Neutral	6.6		Cellular	−	−
16-2	21	None		5.5	Cellular, fibrous	−	−
16-3	23, 25	Acid	73.5	17.8	Cellular, no follicles, intracellular mucin	−	−
16-4	24	Acid			Cellular, no follicles, intracellular mucin	−	−
16-5	23	Acid		6.4	Cellular, no follicles, intracellular mucin	−	−
16-6	18, 19	Neutral			Cellular, follicles with colloid	+	+
Normal thyroid		Neutral		7.7		+	+

* Wollman, S. H. Unpublished data to be reported.

chromatic staining of tumor secretions, it did not alter that in the neighboring mast cells (Figs. 8 and 12).

The material released by mild acid hydrolysis of the tumors—when analyzed by paper chromatography—had the same mobility in two solvent systems as an authentic standard of N-acetylneuraminic acid.

Survey of Tumor Lines

The morphologic characteristics, type of mucin, sialic acid concentration and functional activity of representative tumors in the various tumor lines are listed in Table III. The two measures of functional activity in the tumors were the ability to maintain a concentration of radioiodide elevated above that in the blood serum and the ability to form protein-bound radioiodine (PBI). Those tumors which formed extracellular neutral mucopolysaccharide (tumor lines 1-1C, 1-2) exhibited activity with respect to both concentration of radioiodide and the formation of PBI^{131}. Some of the tumors with extracellular mixed neutral and acid mucins (tumor line 1-8) showed function, and others (line 1-4) did not. Tumors with intracellular acid mucin (tumor lines 16-3, 16-4 and 16-5) or with no mucin (tumor lines 1-1A, 1-3, 1-6, 16-1, 16-2) retained neither biochemical property.

The level of sialic acid in the acid hydrolysates of tumor homogenates generally seemed to agree with the acid mucopolysaccharide staining observed in the histologic sections (Table III). Exceptions were encountered in normal thyroid, which had a considerable concentration of sialic acid and a neutral mucin reaction, and in tumor line 16-2, which had no mucin but possibly contained the sialic acid in fibrous connective tissue.[15]

There was good agreement generally between the estimates of sialic acid in the wet and in the dry tissue obtained from sections of fixed material. The ratio of the concentration in dry tissue to that in wet was ordinarily about 5, the value to be expected if 80 per cent of the fresh tumor were water. The discrepancy in tumor line 1-9 where the ratio was 11.4 correlated well with the observation that in generation 18 the follicles contained colloid, whereas in generation 19 colloid was absent.

Discussion

The acid mucopolysaccharides formed by the rat thyroid carcinomas provide another instance in addition to those already investigated[2] for evaluating the histochemical properties of sialomucins. Thus far these substances have all shown strong Alcian blue affinity, reactivity in the periodic acid-phenylhydrazine-Schiff sequence, azurophilia (usually

metachromatic) at pH 2.5 but not below pH 2, nonreactivity in the periodic acid-diamine procedure, staining with the second of the two basic dyes aldehyde fuchsin and Alcian blue, and reversible loss of the acidic characteristics with mild methylation.

It is noteworthy that the acid mucous secretion in these neoplasms is the only known epithelial sialomucin of the rat in which the purified enzymes from Vibrio and Clostridium have successfully hydrolyzed the sialic acid. The sublingual and submaxillary glands and pregnant vaginal epithelium, which certainly contain sialomucins in the rat, and many laryngotracheal glands which probably do so, resist destruction of basophilia by sialidase. The reason for this resistance to digestion in the normal rat secretions is under investigation. Although as yet unexplained, it appears not to be related to the chemical form of the sialic acid or to the presence of a sialidase inhibitor in the rat tissues.

Sialic acid occurs normally in the thyroid gland in a concentration which exceeds that in tumors containing neutral follicular fluid and equals that in some tumors with basophilic mucopolysaccharide (Table III). However, colloid of the normal thyroid gland lacks the basophilia shown by some secretions in neoplasms. The sialic acid in normal thyroid colloid lacks basophilia possibly because it is masked or bound in some manner. Against this interpretation is the observation that digestion with sialidase releases sialic acid from tissue sections in the normal rat thyroid. Another possible explanation is that the sialic acid concentration is low in normal colloid relative to the basophilic secretion in neoplasms; the amount of colloid in normal glands is proportionately greater than that in follicular tumors.

Somewhat surprising is the existence of acidic mucin in tumors derived from a tissue which normally secretes neutral mucopolysaccharide. Of possible interest in this connection is the fact that epithelium of the primitive pharyngeal anlage in the endostyle of Amphioxus (from which the thyroid gland derives phylogenetically) itself secretes acid mucopolysaccharide and forms organically bound radioiodine.[16]

The correlation of functional activity with the type of mucin secreted by the tumors has been investigated. Although the maintenance of a concentration of inorganic iodide elevated above that in the blood can occur in the apparent absence of colloid (tumor line 1-5A), the formation of protein-bound iodine seems to require the presence of neutral or acid mucin. Preliminary autoradiographic studies indicate that in a neoplasm containing neutral mucins (tumor line 1-1C) all follicles form protein-bound I^{131} as in the normal rat thyroid.[17] On the other hand in a tumor with mixed neutral and acid mucins, some follicles containing acid mucins have no protein-bound radioiodine.

Summary

Follicular fluid in normal thyroid colloid and in some rat thyroid carcinomas contains neutral mucopolysaccharide as shown by histochemical procedures. The follicular fluid or cell cytoplasm of other rat thyroid neoplasms contains abundant mucus showing the histochemical properties of nonsulfated acid mucopolysaccharide. The basophilia of the latter mucin is due to enzymatically hydrolysable N-acetylneuraminic acid. The amount of sialic acid in the tumors varies from less than 30 per cent to several times the concentration in normal thyroid, depending on the line of tumor. The normal thyroid gland contains an appreciable level of sialic acid which for reasons as yet unexplained lacks histochemical basophilia. An attempt is made to correlate functional activity with structure and the type of mucous secretion.

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[*Illustrations follow*]

Legends for Figures

Fig. 1. Normal thyroid showing red staining of neutral mucopolysaccharide in colloid. Alcian blue-periodic acid-Schiff (PAS) stain. × 250.

Fig. 2. Normal thyroid showing nonreactivity of thyroid colloid in contrast to metachromasia of tracheal cartilage. Azure A stain at pH 2.5. × 250.

Fig. 3. Normal thyroid demonstrating orthochromatic staining of colloid. Azure A stain at pH 4. × 250.

Fig. 4. Rat thyroid carcinoma (tumor line 1–2) showing follicular fluid with neutral mucopolysaccharide resembling that in the normal gland. Alcian blue-PAS stain. × 250.

Fig. 5. Rat thyroid carcinoma (tumor line 1–4) exhibiting purple-blue staining of follicular acid mucopolysaccharide. Section incubated with control buffer 16 hours before staining with Alcian blue-PAS stain. × 205.

Fig. 6. A section adjacent to that seen in Figure 5 digested 16 hours with sialidase. Follicular fluid stains like neutral mucopolysaccharide. Alcian blue-PAS stain. × 205.

Fig. 7. Buffer-treated control section adjacent to that shown in Figure 6. There is metachromasia of acid mucin in the follicular fluid. Azure A stain, pH 2.5. × 205.

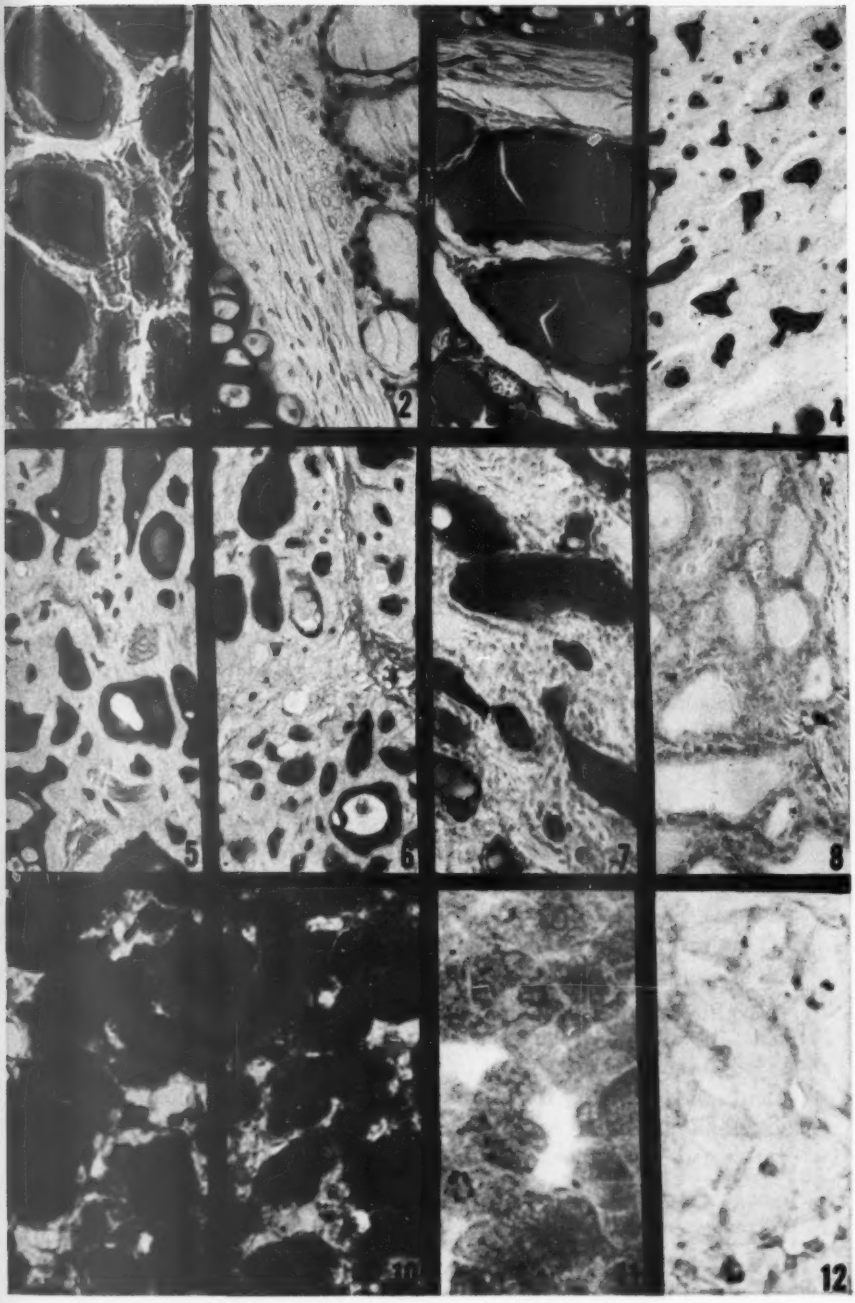
Fig. 8. A section adjacent to that shown in Figure 7 digested 12 hours with sialidase. Note the loss of follicular basophilia. Azure A stain, pH 2.5. × 205.

Fig. 9. Buffer-treated control section of rat thyroid carcinoma (tumor line 16–3). There is purple-blue staining of cytoplasmic acid mucopolysaccharide. Alcian blue-PAS stain. × 450.

Fig. 10. A section adjacent to that shown in Figure 9 digested 16 hours with sialidase. Note loss of basophilia and staining indicative of neutral mucin. Alcian blue-PAS stain. × 450.

Fig. 11. Buffer-treated control section adjacent to that shown in Figure 10. Metachromasia of cytoplasmic acid mucopolysaccharide is manifest. Azure A stain, pH 2.5. × 450.

Fig. 12. A section adjacent to that shown in Figure 11 digested 12 hours with sialidase. Note the loss of basophilia in the cytoplasmic mucin. Azure A stain, pH 2.5. × 450.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REGENERATION OF INJURED AND TRANSPLANTED STRIATED VOLUNTARY MUSCLE IN RATS

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Extensive experimental studies have shown that mammalian skeletal muscles are capable of partial or complete regeneration after certain physical, chemical or mechanical injuries.[1-7] Based on histologic observations of the damaged areas at various intervals, suggestions have been made that the predominant mode of formation of new fibers is through budding of the surviving stumps of muscle fibers,[4-6] although under certain conditions, organization of spindle cells in the area into new fibers has been considered possible.[1-3] In a recent publication, Lash, Holtzer and Swift [7] measured the deoxyribonucleic acid (DNA) content in individual nuclei of regenerating fibers and of mononucleated cells in the injured areas of muscles of mice by a cytophotometric procedure using the Feulgen dye. According to them, nuclei within the regenerating fibers contain a diploid amount of DNA; mononucleated cells in the area also contain multiples of the diploid amount as well as intermediate values. This feature is characteristic of rapidly dividing tissues. From the above findings and the fact that mitotic figures are absent in regenerating fibers, they suggested that mononucleated cells which had undergone a number of mitotic divisions might give rise to newly formed fibers by a fusion process. Amitotic division within the regenerating fibers, as proposed by other authors,[1,4,5] was considered unlikely in this instance because of the lack of synthesis of the genetic material necessary for the functional integrity of the cell.

Since it had been observed, after severe local injury to muscle, that regenerating fibers usually appeared at the peripheral area[4-6] where the trauma was slight, we decided to inflict mild injury and then observe the capacity of newly formed fibers to incorporate radioactive phosphorus (P^{32}) into nucleic acids. In this manner, it would be possible to make comparison of nucleic acid metabolism of this tissue with that of other cells in rapid growth and protein synthesis.[8] In addition, autologous transplantations of muscles to the omental bursas were done to investigate the possibility of re-formation of muscular structures in the absence of intact stumps of muscle.

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Material and Methods

Male Sprague-Dawley rats, weighing 150 to 200 gm., were used throughout the present investigation. In the studies of muscle injuries due to pressure, rats were anesthetized with ether before operation. After skin incision and retraction, the *musculus rectus femoris* was lifted, and a strip, measuring 1 to 2 mm. in thickness, was separated from the belly of the muscle with a sharp scalpel, leaving the ends attached. Pressure was applied on the thin muscle strip by a 5-inch Ralk forceps, which was locked at the first position to exert only a comparatively mild pressure. This procedure was used throughout in order to exclude the variation in thickness of muscles subjected to pressure. The site of injury was marked with a black silk suture (no. 4-0) for identification. The animals were sacrificed by ether at various intervals between 24 hours to 4 weeks. The injured strip of muscle and a similar fragment of normal muscle from the other limb were fixed in acetic acid-ethanol (1:3) or in 10 per cent formalin for histologic examination. After dehydration and embedding in paraffin, sections were cut at 3 to 8 μ. Some of these sections were stained with hematoxylin and eosin or with Hansen's hematoxylin and picrofuchsin.

For the demonstration of ribonucleic acid (RNA), sections of tissues fixed in acetic acid-ethanol were stained with 0.5 per cent toluidine blue in 0.05 M acetate buffer at pH 4. Some of the sections were treated with a 0.5 per cent solution of crystalline ribonuclease (Worthington Biochemical Company) in phosphate buffer at pH 6.8 for 45 minutes at 55° C. before staining. As controls, some sections were treated in the same manner with the buffer alone before staining. Basophilia, which is removable by the enzyme, was assumed to represent the distribution of RNA. DNA was demonstrated by the Feulgen reaction, which involved the hydrolysis of the sections with 1 N HCl at 60° C., washing in distilled water, and immersion in Schiff's reagent for 45 minutes. The dye was washed out twice with a washing fluid containing potassium metabisulfite and HCl.

Some rats were given 100 μc. of P^{32} orthophosphate intraperitoneally 2 days after injury and were sacrificed at various intervals thereafter. Unstained sections prepared from acetic acid-ethanol-fixed tissues were covered with AR-10 stripping film (Kodak Limited, London) under water. Exposures were made at 4° C. for a period of 7 to 21 days. Some sections were treated with ribonuclease before the application of the film. The autoradiographs were developed in D-19 developer (Eastman Kodak Company, Rochester, New York) for 6 minutes, rinsed in 0.6 per cent acetic acid and fixed in Kodak acid fixer for a period of twice the clearing time before washing. A number of sections were stained with 0.5 per cent toluidine blue in acetate buffer at pH 4 after development. Unstained sections were mounted and observed with the phase microscope. In a few instances, sections were subjected to the Feulgen procedure before application of the film.

In the transplantation experiments, fragments of *m. rectus femoris* measuring 1 and 2 mm. in thickness and approximately 5 mm. in length, were removed with a scalpel and grafted into the omental bursa of the same animal. The location of the graft was identified with a silk suture. The grafts were removed at intervals up to 6 weeks after the operation and prepared for histologic observations by the procedures described above.

Results

Alterations Induced by Mild Crushing Injuries

Degeneration. Degenerative alterations observed in the strip of muscle subjected to a mild crushing injury under the present experimental conditions differed to some extent from those observed by other workers

after various types of injury. In sections obtained from animals sacrificed 24 hours after injury, myofibrils were finely fragmented, disorganized and strongly eosinophilic. The stromal framework, on the other hand, retained the characteristic tubular structures of muscular tissue (Figs. 1 and 3). This contrasted with Clark's findings in severely crushed muscles, in which the endomysial stroma was completely distorted.[4,5] Within the tubular structures, which have been called "sarcolemma tubes," there were numerous cells characteristic of phagocytes with single indented nuclei and clumps of acidophilic materials within their cytoplasm (Figs. 1 and 2). In favorable sections, striated fragments of myofibrils were occasionally observed in the cytoplasm. In addition to the cells described above, there were fusiform cells connected by fairly broad bands of cytoplasm [2-4] lying mainly in the peripheral portion of the endomysial tube and oriented in the direction of the original muscle fibers. These cells were readily observable in thicker sections as plasmodia formed by the union of fusiform cells with large nuclei and conspicuous nucleoli (Figs. 1 to 4). In thinner sections, the plasmodia appeared as isolated cells or a string of a few cells connected by protoplasmic strands which could be readily distinguished from fibroblasts in the stroma. In serial sections, many of the fusiform cells were connected by bridges to form plasmodia in the majority of the muscles observed. The plasmodia were distributed mainly in the peripheral area of the injured portion of the muscle, although a number of them could also be seen in the central region. The interconnected cells have been observed by a number of authors.[4-6] The consensus is that they represent the surviving portion of muscle fibers after demyofibrillation. This was supported by the fact that they maintained the elongated, ribbonlike shape and general distribution resembling early regenerating fibers to be described in the next section. Additional evidence was their appearance only in injured muscle and not in wounds of skin, connective tissue or visceral organs.

The tubular stroma containing cells as described has been called "muscle cell tube" or "sarcolemma tube." The former term was first used by Waldeyer [9] in the belief that all cells within these structures were muscular in origin. That this is not an accurate conception can be readily realized by the observation of erythrocytes, neutrophils and other cells of nonmuscular origin within the tubes.[2,3] The term "sarcolemma tube" suggests that the tubular framework as seen in these lesions represents the original envelope of the muscle fiber. However, various definitions have been given to the term sarcolemma, as clearly discussed by Bennett.[10] According to him, the term should be restricted to the plasma membrane of the muscle cell. In the present article, these tubular structures will be

considered as endomysial tubes, indicating that they are connective tissue boundaries within which remnants of degenerated materials, cells of various types, as well as free tissue fluid, are present. It can be seen clearly in histologic sections that phagocytic cells are free within the endomysial tubes without actual invasion into the remainder of the sarcoplasm as suggested by some authors.[7]

Under the present conditions, phagocytic removal of the degenerated myofibrils in the injured area was completed within 48 to 72 hours, leaving the tubular stroma intact (Figs. 3 and 4). Strands of multinucleated fusiform cells were well preserved in most areas, but they appeared to be more numerous in the peripheral region. During the entire period of observation, morphologic signs of nuclear destruction, such as karyorrhexis, pyknosis and karyolysis were minimal. Infiltration of the damaged area by granular leukocytes was observed occasionally, shortly after the injury, but they were very few in number in most cases.

The alterations described above involved mainly the destruction of the contractile structure, with minimal loss of muscle nuclei and paranuclear cytoplasm. Thus it corresponded to the condition called "dissociative degeneration," described by Pfuhl[11] and others[4-6] in areas between total destruction and the intact stumps of muscles in the more severe forms of injuries.

Regeneration. The process of regeneration did not differ to any considerable extent from that observed by other authors[1,6] except that the time required for various stages was considerably shorter. Bands of regenerating fibers with centrally located nuclei were demonstrable as early as 48 hours after the injury, a stage in which removal of the degenerated myofibrils was still in progress. These bands appeared like basophilic ribbons containing large nuclei and prominent nucleoli, as described by others.[7] They were called "sarcoblastic ribbons" by Betz[12,13] and Godman[6] and "myotubes" by Lash, Holtzer and Swift.[7] The fact that they are regenerating fibers has been demonstrated by the observation of myofibrils in these structures during the later stages.[6,7] There may be more than two of these regenerating fibers within one endomysial tube. The regenerating fibers were distributed predominantly in association with the intact stumps of the fibers in the periphery of the injured area (Figs. 3 to 5). Thus our findings were essentially in agreement with those of Clark[4,5] and Godman.[6] However, examinations of serial sections and of thick sections did not indicate a direct connection between the regenerating fibers and the surviving stumps to any observable extent. The basophilic regenerating fibers were usually located laterally to the surviving fibers without any intimate sarcoplasmic connection in most areas, while in areas where the surviving fibers were absent, they appeared to be

independent (Fig. 4). Mitotic figures were not observable in these newly formed fibers in the 20 animals examined, although they were fairly numerous in cells lying free within the endomysial tubes. The identification of these mitotically active cells with muscular or connective tissue elements was difficult because of the lack of a definite morphologic criterion, although their cytoplasm was more basophilic than that of fibroblasts and phagocytes, as noted by Lash and co-workers.[7] Later stages in the development of regenerating fibers involved the new formation of myofibrils and the displacement of nuclei toward the periphery. This has been described in detail by others.[6,7] At the end of one month after injury, the regeneration of striated muscles was almost complete, except in a very few animals whose operative wounds were heavily infected. Multinucleated muscle giant cells of irregular contour, usually seen in more severe injuries in areas where the tubular stroma was destroyed, were not present in any of the sections examined in the present series.

The Incorporation of P^{32} into Nucleic Acids of Regenerating Fibers

In the cytochemical observations of the distribution of ribonucleic acid, toluidine blue in acetate buffer at pH 4 was used for staining sections obtained from acetic acid-ethanol-fixed tissue. This procedure was used to minimize nonspecific staining of proteins as suggested by Herrmann, Nicholas and Boricious in their investigation of the developing muscles of the uterus.[14] Under this circumstance, intact muscles stained only within their nuclei, while sarcoplasm and myofibrils were very slightly colored or completely unstained. The regenerating fibers, on the other hand, exhibited an intense basophilia (Fig. 6). In sections previously treated with ribonuclease, only nuclear basophilia remained, suggesting that the concentration of RNA within the sarcoplasm of these regenerating fibers was relatively high (Fig. 7).

In rats given P^{32} as inorganic phosphate during the first 2 days after injury and sacrificed 2 days later, autoradiographs of the regenerating areas also showed dense silver grains over the regenerating fibers (Figs. 8 and 9). Some incorporation was present over the surviving stumps when compared with the background region. These features were absent in autoradiographs of sections which had been treated with ribonuclease. The distribution of RNase-sensitive grains was considered to represent the approximate distribution of RNA within the muscle fibers.

The distribution of P^{32} incorporated into DNA was determined in sections treated with ribonuclease and in those subjected to the Feulgen procedure before the application of stripping film, following the methods employed by Leblond, Stevens and Bogoroch[15] and by Howard and

Pelc.[16] Since the radioactive compound was given on the second day, at the beginning of the rapid appearance of regenerating fibers, it was hoped that incorporation would occur if there was an active synthesis of DNA. In animals sacrificed 2 to 7 days after injections of the isotope, examination of autoradiographs did not show any significant number of silver grains over nuclei of sarcoblastic fibers or intact muscle above that of the background. This suggests that the incorporation of P^{32} into DNA occurred only very infrequently or that it was absent in these nuclei. A number of nuclei in the phagocytes, on the other hand, showed evidence of incorporation.

The Reactions of Striated Muscles to Omental Transplantation

Although mild injuries do not completely destroy all structures within muscle fibers, it was found that regenerating fibers still appeared mainly in association with the surviving stumps, as in the more severe type of injuries. Under the conditions of the experiment, however, it is conceivable that the center of the lesion was still more severely damaged, although this was not readily demonstrable morphologically. If this were the case, the initial formation of sarcoblastic fibers would involve the portions of fibers that were less severely affected functionally. It appeared that by transplantation of the muscle to another site it might be possible to induce a different type of injury and also to determine whether regeneration was possible under this condition. By cutting a fragment of muscle with a minimal amount of crushing pressure, the injury was inflicted mainly on the periphery of the tissue mass. After transplantation, on the other hand, further damage could occur by the initial lack of blood supply. Under this condition, it was expected that the central portion would be more severely affected. By using extremely thin grafts, the second effect could be minimized. It was necessary to observe degenerative changes during the initial period in order to assess the degree of damage and the distribution of lesions as well as to exclude the possibility of the survival of certain portions of muscle fibers in a morphologically intact condition.

Degenerative alterations of the omental graft were similar to those in the locally injured muscle, but there were certain significant differences. Within the endomysial tubes of the graft, myofibrils assumed the appearance which is usually described as conchoidal or discoidal degeneration.[1,4] This consisted of an accentuation of cross striations with retention of anisotropic discs across the width of the fiber. Individual myofibrils, on the other hand, seemed to be broken up at regular intervals in the region of the isotropic bands, resulting in what is usually described as a loss of longitudinal striations. The affinity of the contractile structure for eosin was markedly increased. Muscle nuclei were present and appeared in-

tact in most of the areas examined, although in some thicker grafts, karyorrhexis and pyknosis were observable in certain regions. Within 12 to 24 hours, phagocytes could be seen readily in the more superficial portion of the graft within endomysial tubes which contained eosinophilic or hyaline debris resulting from a complete dissolution of the contractile structure. Toward the center of the transplant, on the other hand, anisotropic or conchoidal discs were fully intact (Fig. 10). In addition to phagocytes, bands of basophilic plasmodia, described in the previous section as representing the remains of the sarcoplasmic and nuclear components of the muscle fibers, were more conspicuous than generally seen in locally injured muscles. During the period between 2 to 5 days after the operation, invasion by phagocytes continued toward the center of the graft, while endomysial tubes in the peripheral region became free of degenerated materials. It is remarkable that the cross striated discs in the central region retained their morphologic characteristics for as long as 5 days in the center of the graft. However, they eventually disintegrated during the second week. The alterations were similar to those observed in ischemic muscles by various workers [1,4,5] and investigated in detail more recently by Harman [17] in experimental animals after the application of tourniquets to the limbs.

The process of regeneration in the graft was demonstrable as early as 48 hours after the operation. Numerous regenerating fibers were distributed in the peripheral region within the endomysial tubes (Fig. 11). In some areas they appeared even before complete removal of the myofibrillar debris. In the center of the graft where conchoidal discs still remained, no regenerating fibers were visible. After a period of 4 to 6 weeks, muscle fibers containing cross striated myofibrils and subsarcolemmal nuclei were numerous. These fibers were smaller in diameter than those of normal muscle, and the endomysial and perimysial connective tissue was dense, especially in the periphery of the transplant (Fig. 12).

A total of 20 transplantations were performed; 14 of these showed the morphologic features described. In 4, there were marked neutrophil infiltrations and extensive destruction of the muscle without any evidence of regeneration. In the remaining animals, the process of regeneration was abortive, with a dense encapsulation of connective tissue around the grafts.

Thus muscle isolated in omental bursas could regenerate into fibers with mature characteristics in complete separation from the surviving stumps of intact fibers, although the duration of survival of these differentiating characteristics beyond 6 weeks was not determined. These results are to be compared with earlier studies of muscle grafts for the

solution of other problems [12,18,18] in which isolated cells or spindle cells lacking in these characteristics appeared. The difference in results can be accounted for by the fact that in these studies either a mince of muscle or exceedingly large pieces were grafted into areas with relatively poor blood supply. Under the conditions in our experiments, fragments of muscles exceeding 4 mm. in thickness also failed to form myofibrils during the later stages of regeneration.

Discussion

Under the present experimental conditions, mild pressure injury to skeletal muscle of the rat was followed by increased growth of new fibers, resulting in an almost complete regeneration within a short period. This observation suggests that with a mild injury, structures essential for regeneration were not destroyed to the same extent as in severe trauma. This hypothesis was strengthened by the observation that numerous plasmodia of cells of apparent muscular origin survived in large numbers. The degree of damage in this case was comparable to that observed by other authors in the peripheral area of lesions. However, there was still a gradient of severity from the center toward the periphery of the lesion, as indicated by the fact that regenerating fibers were more numerous in the latter area. Direct budding from intact stumps of muscle fibers was not essential for the regenerative process because many regenerating fibers grew without any connection with intact fibers in mild pressure injury and in omental grafts. In the latter case, new fibers appeared more rapidly in areas where myofibrils had been removed most rapidly by phagocytes.

As described by Lash and co-workers,[7] the newly formed fibers contained enlarged nucleoli and a high concentration of RNA, especially in the paranuclear region. These are features characteristic of cells in the process of regeneration or in the rapid synthesis of proteins.[7] Our autoradiographic findings supplement the above data with the fact that these structures could actively incorporate P^{32} into their RNA in high concentration. However, high RNA concentration and incorporation of P^{32} into the nucleic acid as observed by the present methods might still be open to a second interpretation. If the contractile structure is more susceptible to injury, RNA-containing structures might survive to a greater extent and become condensed into a more compact mass during the reduction in size of the fiber which had been transformed into elongated plasmodial strands. Under these circumstances, cytochemical as well as autoradiographic studies of RNA would indicate high concentration. That this occurred to some extent was possible. However, when con-

sidered together with other morphologic features, the conclusion reached by Lash and co-workers,[7] that these regenerating fibers represented typical regenerating cells, appears valid.

The incorporation of P^{32} into DNA of the nuclei of regenerating fibers, on the other hand, was not demonstrable by the present procedure, in spite of the fact that the isotope was given during a period when numerous new fibers were appearing. A number of mononucleated cells in the area, however, were capable of DNA synthesis to a conspicuous degree. This finding, together with the fact that nuclei of regenerating fibers are mainly diploid,[7] tends to indicate that proliferation of nuclei within the fibers is infrequent or absent during the rapid appearance of regenerating fibers. It also tends to exclude the possibility of fusion of mononucleated cells into new fibers, as suggested by Lash and associates,[7] because, were this the case, radioactive DNA should have been carried into the fibers during the fusion process because of the stability of the compound. Thus, the findings give rise to a conclusion which is apparently in contradiction to the reported increase in nuclear number.

Although estimations of nuclear number have been made,[7] the values reported represent only nuclear density in sectioned fibers. To establish that there was an actual increase, nuclear number in whole fibers would be necessary. It is, however, very difficult to obtain these values because regenerating fibers are easily torn during the teasing procedure. Examination of teased normal fibers indicates that muscle nuclei, distributed under the sarcolemma, are more numerous than is apparent in histologic sections which include only a fraction of the fiber. In atrophic or small fibers, more nuclei can be observed in sections although the total number in whole fibers does not exceed that of fibers of average size, because a larger fraction of the fiber is included in the section. Thus, nuclei observed in regenerating fibers might derive from those present in the original mature fibers which had undergone differential destruction of the contractile component. The above consideration, together with the fact that nuclei in certain regions of a muscle fiber have been observed to pass from an uninjured area to the degenerated region in muscles of living tadpoles [19] is highly suggestive that there is no conclusive evidence of nuclear proliferation during early regeneration.

The above facts suggest that following a mild injury, muscle fibers suffer the loss of their contractile structure, but their nuclei and sarcoplasm survive to form elongated plasmodia which later reform myofibrils. The process of regeneration thus might involve mainly the reformation of intracellular structures, rather than the formation of new cells, as observed in the liver and other organs.

Summary

1. After a mild pressure injury, mammalian striated muscle fibers lost their contractile myofibrils, but most of their sarcoplasmic and nuclear components survived as elongated plasmodia.

2. The process of regeneration after such trauma began as early as 48 hours after the injury and progressed much more rapidly than previously observed in more severe conditions.

3. After intraperitoneal injections of P^{32}, regenerating fibers rapidly incorporated the isotope into their RNA. The isotope was not incorporated into nuclear DNA of the newly formed fibers during their appearance although it could be demonstrated in mononucleated cells in the injured area.

4. Autologous transplantation of thin pieces of skeletal muscles into the omental bursa was followed by initial degeneration and subsequent growth of muscle fibers. These regenerating fibers were able to form new contractile structures and assumed a mature appearance. The grafts survived as differentiated muscles for at least 6 weeks. This indicates that budding from intact stumps of muscles is not essential for the process of regeneration.

5. It is suggested that regeneration in mammalian skeletal muscles involves principally the reformation of myofibrils in partially injured fibers without actual proliferation of muscle nuclei.

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[*Illustrations follow*]

Legends for Figures

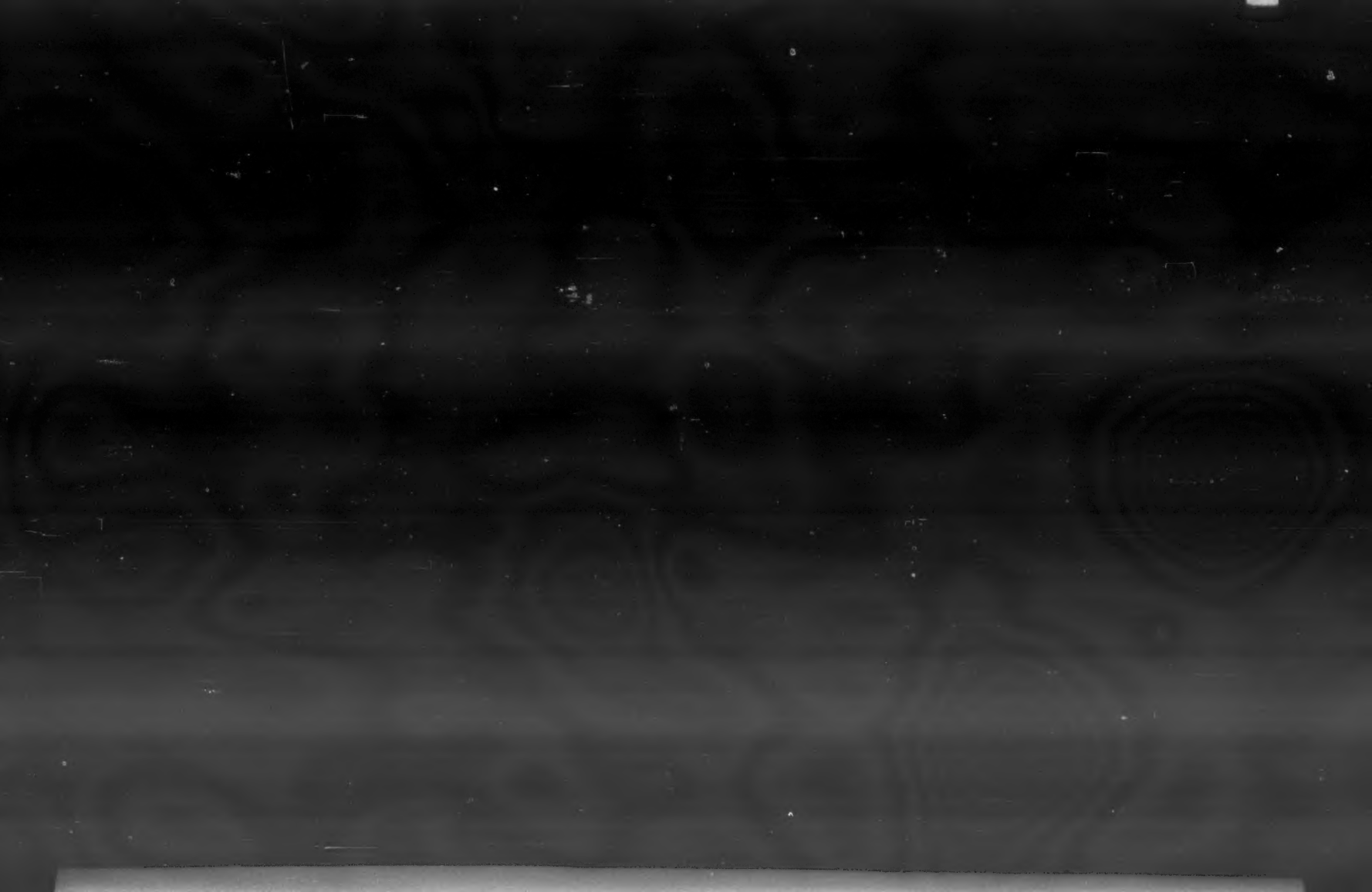
Except where indicated the sections photographed were stained with hematoxylin and eosin.

Fig. 1. Crushed muscle 24 hours after injury. Tubular stroma is filled with phagocytes. Spindle cells and plasmodia represent surviving portions of muscle fibers (arrows). × 175.

Fig. 2. A higher magnification of crushed muscle 24 hours after the injury. Endomysial tubes contain degenerated myofibrils, phagocytes and muscle plasmodia (arrows). × 460.

Fig. 3. An area adjacent to the surviving stump of a crushed muscle 48 hours after injury. Note well preserved stroma in the damaged area and the completion of the phagocytic removal of degenerated myofibrils. The arrow indicates a regenerating fiber. × 175.

Fig. 4. A well preserved area of muscle stroma in the central region of an injured muscle 48 hours after injury. The arrow indicates a muscle plasmodium during an early stage of regeneration. × 460.



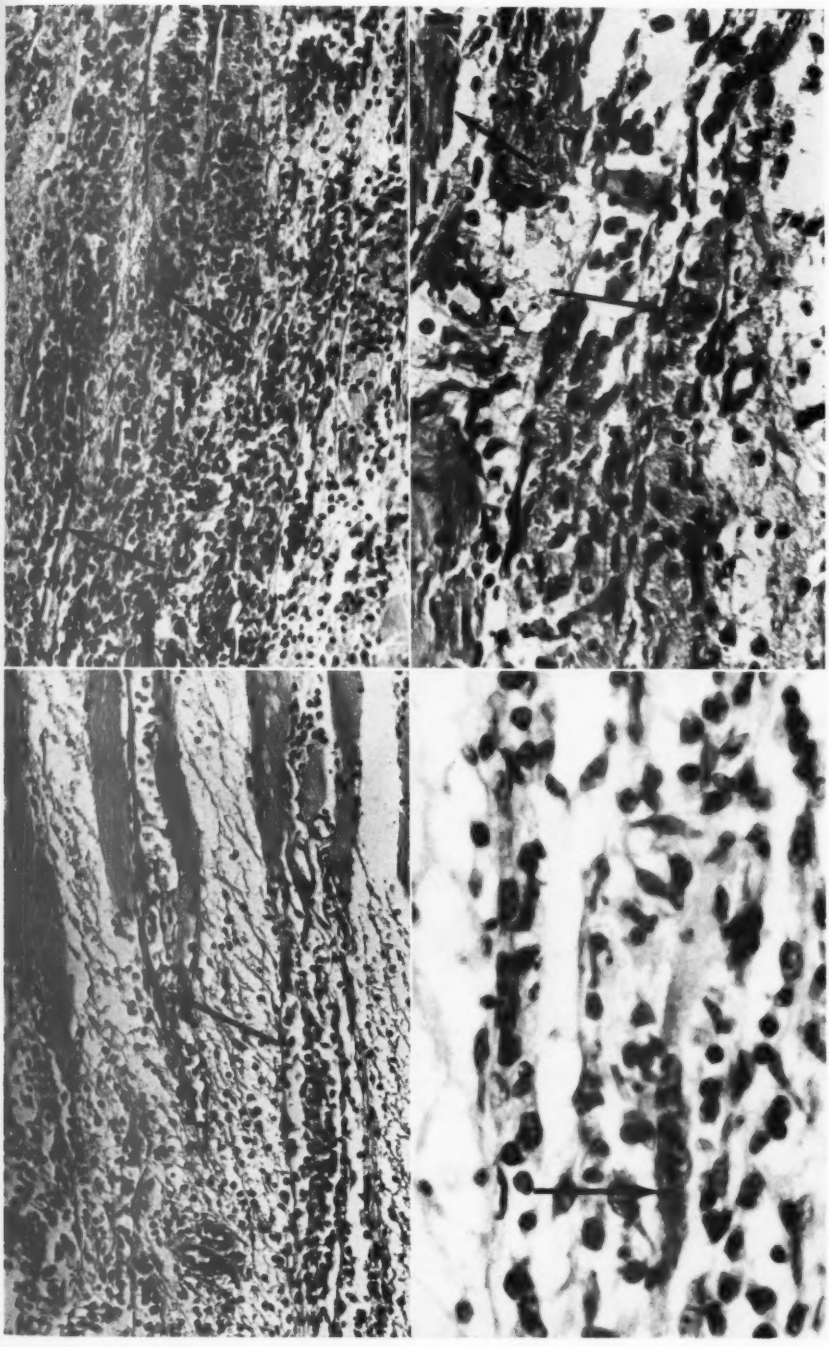

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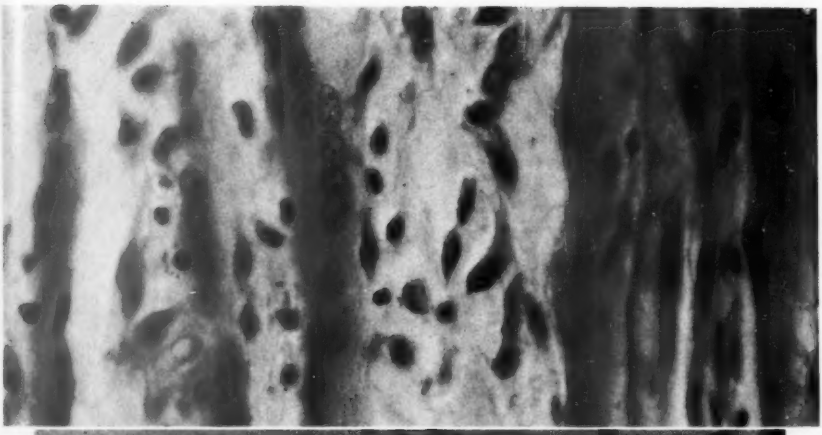
FIG. 5. An area adjacent to the surviving stump of a crushed muscle, showing many regenerating fibers on the fourth day after injury. Some of the fibers contain a few contractile fibrils. × 460.

FIG. 6. Regenerating fibers in a crushed muscle 3 days after injury (arrow) showing affinity for a basic dye. Toluidine blue stain. × 460.

FIG. 7. Regenerating fibers, showing the loss of sarcoplasmic basophilia. Treatment with ribonuclease before staining. Toluidine blue stain. × 460.

FIG. 8. A P^{32} autoradiograph of a regenerating fiber and a surviving stump (lower). Note the density of photographic grains over the regenerating fiber. Unstained. × 990.

FIG. 9. The same area shown in Figure 8 as seen with a phase microscope. The regenerating fiber is evident above an intact stump. Unstained. × 990.



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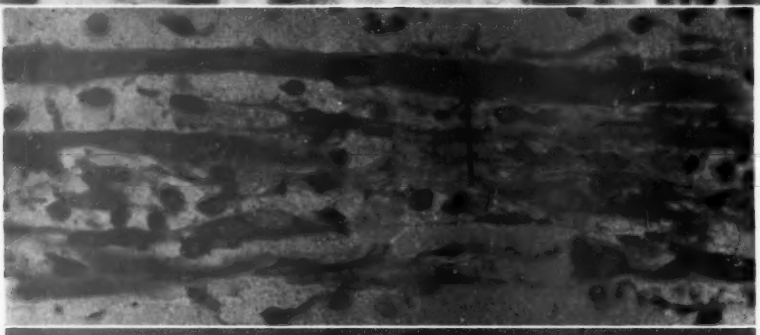

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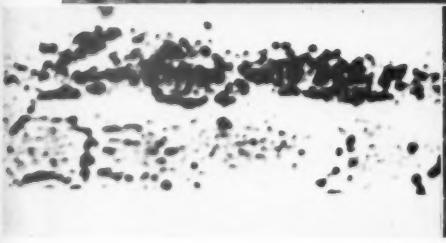

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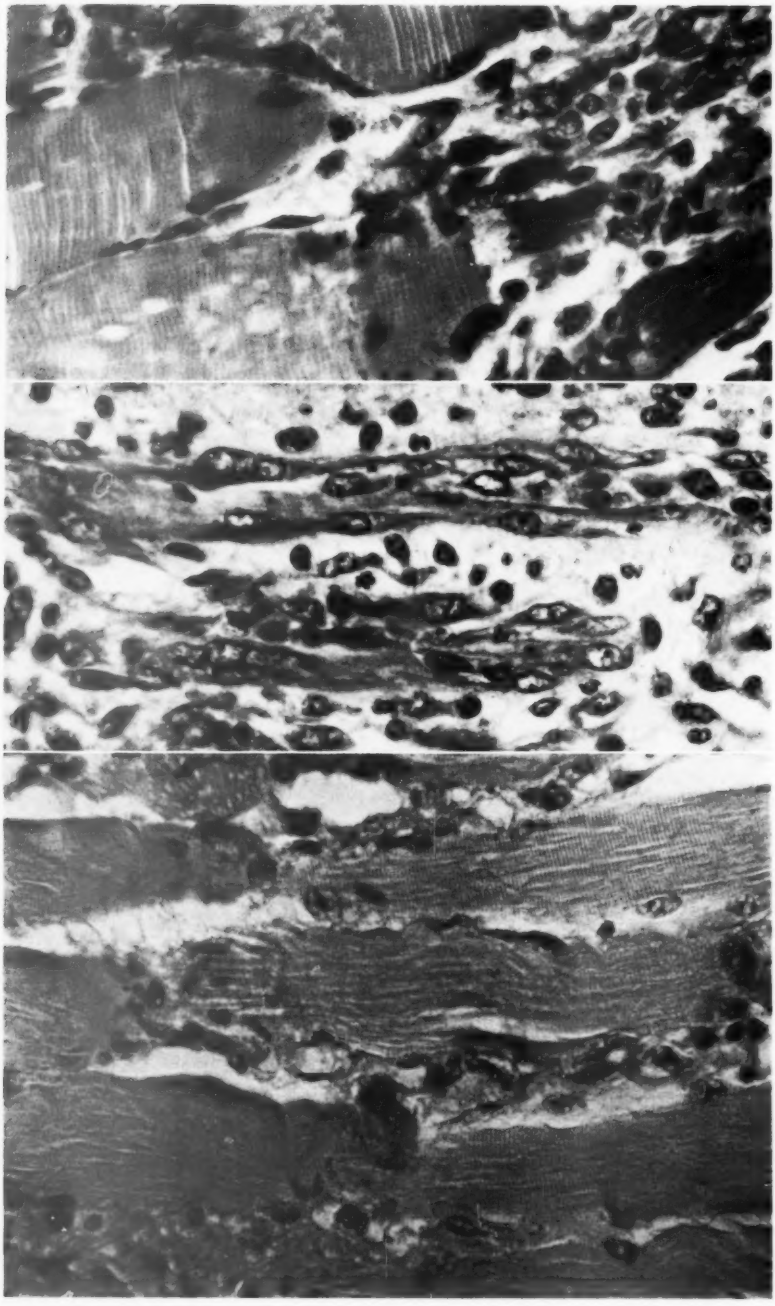
FIG. 10. An omental transplant of muscle 24 hours after the operation. Note discoidal degeneration of the contractile structure. × 650.

FIG. 11. Numerous regenerating fibers in an omental transplant 3 days after the operation. × 650.

FIG. 12. A longitudinal section of a muscle transplant after 6 weeks. × 650.



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EARLY PATHOLOGIC AND BIOCHEMICAL CHANGES IN RABBITS FED DIHYDROCHOLESTEROL

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Previous studies[1] have shown that rabbits fed 0.25 to 1 per cent dihydrocholesterol (3β-cholestanol) for 3 to 6 weeks consistently develop gallstones and an inflammatory reaction in the biliary tract. In these experiments 3 animals received dietary dihydrocholesterol for 1 week only, and necropsy revealed an inflammatory reaction of the biliary tract but no concrements in the gallbladder.

In the present study the early phases of the experimentally induced disease were investigated in order to ascertain: (a) whether the inflammatory reaction constituted the first stage of the disease; and (b) whether the onset and development of the disease could be related to biochemical alterations in the composition of bile, gallstones or serum and liver sterols.

METHODS

Forty-five male chinchilla and albino rabbits were kept in individual cages and were fed a diet consisting of Purina Rabbit Chow pellets plus 12 per cent olive oil. Dihydrocholesterol (0.5 per cent by weight; generously supplied by the Schering Corporation) was incorporated into this diet as previously described.[1] Groups of 3 to 8 rabbits were sacrificed by intravenous injection of pentobarbital after 1 to 18 days' dihydrocholesterol feeding. The time intervals employed and the number of animals in each group are listed in the first two columns of Table I.

The intensity of the gross and microscopic lesions in the gallbladder and in the intra- and extrahepatic bile ducts was graded as follows: 0 (no lesions) to ++++ (very severe lesions). The following findings were graded by this method: (a) cellular reaction and fibrous thickening of the gallbladder, extrahepatic bile ducts, and intrahepatic bile ducts; (b) presence of concrements in the gallbladder, extrahepatic bile ducts, intrahepatic bile ducts, and common duct.

The weight of the gallstones was determined after drying them in a vacuum oven at 60° C. Aliquot samples of serum and liver were hydrolyzed with alcoholic potassium hydroxide. The nonsaponifiable fraction was extracted with n-hexane and analyzed for total sterol by digitonin precipitation and for cholesterol by the method of Schoenheimer and Sperry.[2] The difference between the total sterol concentration and the cholesterol concentration was assumed to be due to the presence of the saturated sterol, dihydrocholesterol.

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The pH of the gallbladder bile was determined as soon after sacrifice as possible, using a one-drop glass electrode (Beckman #40278) and a calomel reference electrode in a Beckman Model G pH meter. The total solid content of the bile was determined by drying a suitable aliquot in a vacuum oven at 60° C. The concentration of cholic acid and deoxycholic acid in the bile were measured spectrophotometrically.[4] The dried biliary concrements were powdered with mortar and pestle and 10 mg. portions were hydrolyzed in 10 ml. of 10 per cent aqueous NaOH in an autoclave (14 pounds per square inch) for 3 hours. The hydrolysates were assayed for cholic and deoxycholic acids by the procedure used for the bile.[4] Dihydrocholesterol-4-C^{14} was prepared by the catalytic hydrogenation of cholesterol-4-C^{14}.[5]

Sections of liver, gallbladder and extrahepatic ducts were fixed in formalin and Zenker's solution. Paraffin sections of material fixed in Zenker's solution were prepared and stained with hematoxylin and eosin. Special stains were employed to elucidate certain features when necessary. Frozen sections of formalin-fixed tissues were cut at 15 μ and stained with oil red O.

Results

Table I shows the pathologic involvement of the biliary tract and the average dry weight of the gallstones during certain stages in the course of the experiment. The values for the severity of the inflammatory reaction and of the lithiasis have been listed separately in order to give a more complete picture of the relative contribution of these factors to the total pathologic involvement. During the first 4 days of the experiment,

Table I

PATHOLOGIC INVOLVEMENT OF BILIARY TRACT IN RABBITS FED 0.5 PER CENT DIHYDROCHOLESTEROL

Days on regimen	No. of animals	Av. dry wt. of gallstones (mg.)	A: No. of animals with		B: Average severity of	
			Inflammatory reaction	Biliary concrements	Inflammatory reaction *	Cholelithiasis *
0	5	0	0	0	0	0
1	3	0	1	0	++	0
2	3	0	1	0	+	0
4	3	0	1	0	+	0
5	5	0	4	0	++	0
7	7	6	6	2	++	+
9	7	57	6	4	++	+
11	8	69	7	6	+++	++
14	3	76	3	3	++++	++
16	3	176	3	3	+++++	+++
18	3	238	3	3	++++++	+++

* In the animals listed in column A. Severity of lesions graded as described in section on Methods; plus signs indicate the average involvement in 3 sites: gallbladder, extrahepatic bile ducts and intrahepatic bile ducts. The maximal involvement by inflammatory reaction = 12+; by cholelithiasis = 16+.

dihydrocholesterol feeding produced a minimal inflammatory reaction in the biliary tract in 3 of 9 animals studied. On the fifth day 4 of the 5 rabbits in this group had minimal inflammatory changes in various parts of the biliary tract, but no gallstones were seen. At the end of 7 days, con-

crements weighing 14 and 30 mg. were found in the gallbladders of 2 of the 7 rabbits examined. At this time 6 of the 7 rabbits exhibited minimal though definite histologic damage of the biliary tract (Fig. 1). These findings suggest that the inflammatory reaction tends to precede the formation of the biliary calculi.

After 7 days the average pathologic involvement increased in a fairly regular manner; all of the animals sacrificed after 11 days had gallstones

TABLE II

ANALYSIS OF GALLBLADDER BILE IN RABBITS FED 0.5 PER CENT DIHYDROCHOLESTEROL

Days of experiment	No. of animals	Total solids (mg./ml.)	Total bile salts * (mg./ml.)	pH
0	5	136	96.1	7.24
1	3	50	35.2	8.24
2	3	102	64.4	7.49
4	3	123	73.7	7.15
5	5	99	53.3	7.58
7	7	128	76.9	7.39
9	7	101	74.9	7.74
11	8	120	72.9	7.34
14	3	Not determined	42.8	8.15
16	3	98	63.1	7.46
18	3	96	71.6	7.36

* Calculated as the glycine conjugates.

and exhibited an inflammatory reaction in the biliary tract. At the end of 18 days the extent of pathologic involvement and the average weight of the gallstones were similar to those seen in animals after a 28 day feeding experiment [2] (Fig. 2).

Table II shows the average concentration of total solids, total bile acids, and the pH of the gallbladder bile during the experiment. On the average, the solid content of the bile samples taken during dihydrocholesterol administration was less than that of the controls. Therefore, the precipitation of the biliary concrements cannot be ascribed to an increase in the concentration of biliary constituents. There was no explanation for the large decrease in the concentration of total solids and of bile salts, and the increase in pH of the bile of animals killed after 1 day and after 14 days of dihydrocholesterol administration.

Table III presents the bile acid concentration of the bile and of the gallstones during the course of the experiment. In normal rabbit gallbladder bile, deoxycholic acid is the major cholanic acid constituent, cholic acid amounting to less than 10 per cent of the total. Thus the weight ratio of deoxycholic acid to cholic acid is 12.9. This ratio decreased fairly regularly during the experiment (except on day 5), attaining a value of 6.9 on day 18. The table further shows that on a dry weight basis 70 to

TABLE III

COMPOSITION OF BILE AND GALLSTONES IN RABBITS FED DIHYDROCHOLESTEROL

	Bile					Gallstones					
		DCA *	CA †	TBA ‡				DCA *	CA †	TBA ‡	
Days on regimen	No. of samples	as glycine conjugates (mg./ml.)			DCA/CA ratio	No. of samples	Average dry wt.	as glycine conjugates (mg./gm. gallstone)			DCA/CA ratio
0	4	89.2	6.9	96.1	12.9		0				
1	2	32.8	2.4	35.2	13.8		0				
2	3	58.9	5.5	64.4	11.7		0				
4	3	66.5	7.2	73.7	9.3		0				
5	4	49.6	3.7	53.3	13.9		0				
7	6	67.7	9.2	76.9	8.4		6				
9	4	66.4	8.5	74.9	9.2	3	57	633	53.7	687	11.7
11	4	65.7	7.2	72.9	8.7	2	69	613	70.4	683	8.6
14	2	38.1	4.7	42.8	7.9	3	76	696	51.2	747	13.4
16	1	55.7	7.4	63.1	7.7	3	176	746	59.7	806	12.4
18	2	61.8	9.8	71.6	6.9	3	238	704	68.0	772	10.2

* DCA = deoxycholic acid.
† CA = cholic acid.
‡ TBA = total bile acid.

80 per cent of the gallstones consisted of bile salts. The sterol concentration was less than 1 per cent in all of the samples investigated, and has not been included in the table. Presumably inorganic salts and bile pigment (biliverdin) made up the remainder of the concrements.

Table IV shows the average changes in serum and liver sterol concentrations during this study. Although the total sterol concentration of serum and liver began to rise only after 4 to 5 days of dihydrocholesterol

TABLE IV

AVERAGE SERUM AND LIVER STEROL CONCENTRATIONS OF RABBITS RECEIVING 0.5 PER CENT DIETARY DIHYDROCHOLESTEROL *.

		Serum				Liver			
Days on regimen	No. of animals	Total sterol (mg.) ‡	Cholesterol (mg.) ‡	DHC † (mg.) ‡	DHC %§	Total sterol (mg./gm.) ¶	Cholesterol (mg./gm.) ¶	DHC † (mg./gm.) ¶	DHC %§
0	5	44.1	39.8	4.3	9.8	2.90	2.34	0.56	18.6
1	3	60.1	38.2	21.9	36.4	2.25	1.48	0.77	34.2
2	3	37.6	26.7	10.9	28.9	2.03	1.64	0.59	29.1
4	3	119.3	82.2	37.1	31.1	3.17	2.38	0.79	24.9
5	5	134.6	95.6	39.0	29.0	5.40	2.58	2.82	52.3
7	7	100.3	58.0	42.3	42.2	4.45	3.02	1.43	32.2
9	7	110.9	49.0	61.9	55.8	6.65	3.66	2.99	45.0
11	8	107.1	51.8	55.3	51.6	4.97	3.10	1.87	37.6
14	3	156.8	51.7	105.1	67.0	5.30	2.66	2.64	49.8
16	3	113.7	41.2	72.4	63.7	7.23	3.20	4.03	55.8
18	3	116.6	42.8	73.8	63.3	4.83	2.20	2.63	54.4

* Basic diet: Purina Rabbit Chow pellets plus 12 per cent olive oil, U.S.P.
† DHC = dihydrocholesterol.
‡ Per hundred cc. of serum.
§ Per cent dihydrocholesterol in total sterol fraction.
¶ Mg. per gm., wet weight.

feeding, the average dihydrocholesterol content of the serum total sterols rose from its control value of about 10 per cent to 36 per cent during the first day of the experiment, and remained close to 30 per cent for the next 4 days. The dihydrocholesterol content of the total sterol fraction then rose steadily to its maximal value of about 65 per cent. The dihydrocholesterol content of the liver followed a similar pattern. In general, serum and liver cholesterol concentrations remained close to normal values during this experiment so that the rise in total sterol concentration was largely due to an accumulation of dihydrocholesterol.

Table V illustrates the specific radioactivity of total sterols in plasma, liver and muscle and of the bile acids isolated from the biliary concrements of a rabbit which had been fed a diet containing 1 per cent dihydrocholesterol-4-C^{14} for 4 weeks. This table shows that about 50 per cent of the cholanic acids present in the gallstones were derived from labeled dihydrocholesterol, since these acids were found to have about one half the specific radioactivity (64 c.p.m. per mg.) of the administered dihydrocholesterol (119 c.p.m. per mg.). It has been previously shown that dihydrocholesterol is not converted to cholesterol in the rabbit.[6] Consequently, the radioactivity of the bile acids in the gallstones cannot be ascribed to the reaction sequence: dihydrocholesterol → cholesterol → cholanic acid.

TABLE V

RADIOACTIVITY OF TISSUE STEROLS AND CHOLANIC ACIDS IN A RABBIT FED DIHYDROCHOLESTEROL-4-C^{14} *

Substance	Specific radioactivity (c.p.m./mg.)
Dietary dihydrocholesterol	119
Gallstone cholanic acids	64
Plasma total sterols	67
Liver total sterols	60
Muscle total sterols	46

* This animal received 1 per cent dihydrocholesterol-4-C^{14} in its diet for a period of 4 weeks.

DISCUSSION

Dihydrocholesterol is absorbed very readily by the rabbit. After feeding a diet containing 0.5 per cent of this sterol for 1 day, nearly ⅛ of the serum and liver sterols consisted of dihydrocholesterol. Nevertheless, about 1 week's dihydrocholesterol feeding was required to produce the first recognizable pathologic alteration in the biliary tract. This observation suggests that it is a metabolite of dihydrocholesterol, rather than dihydrocholesterol itself which is responsible for the irritation of the

biliary tract and the formation of concrements. The nature of this metabolite may be inferred from 2 observations reported in this study:

First, the gallstones contained less than 1 per cent sterol and consisted largely of bile salts resembling, but not identical with, glycodeoxycholic and glycocholic acids.

Second, in the feeding experiments with labeled dihydrocholesterol, the specific radioactivity of the bile acids in the gallstones was approximately the same as that of the mixture of cholesterol and dihydrocholesterol in liver and serum. Therefore, it seems likely that the cholanic acids in the gallstones were derived about equally from endogenous cholesterol, giving rise to 5β-cholanic acids, and from exogenous dihydrocholesterol, leading to the production of 5α-cholanic (allocholanic) acids. Other investigators [7,8] have shown that in the rat dihydrocholesterol is converted to bile acids which differ from the naturally occurring cholanic acids. They have suggested, but have not proved, that these are 5α-cholanic acids. This hypothesis is supported by our observations [6] that the crystalline glycine conjugate isolated from the gallstones differs from known glycodeoxycholic acid in certain physical properties (melting point, optical rotation, infra-red spectrum).

It was not possible to correlate the formation of the biliary concrements with changes in the pH, in the composition of the bile, or in the composition and concentration of tissue sterols. It was observed, however, that the proportion of cholic acid in bile increased during the course of the experiment, while the composition of the gallstones did not change in a similar manner. This finding cannot be explained in terms of our present knowledge. The difference between the deoxycholic acid to cholic acid ratio in bile and concrements from the same animal may be due to the fact that bile acids in bile represent cholanic acids recently supplied by the liver, while bile acid composition in the concrements is an average value produced during the course of days or weeks.

The results obtained in the present study indicate that the inflammatory reaction precedes or coincides with the appearance of biliary concrements. It would appear that the inflammatory reaction of the gallbladder is not caused by the presence of a few small concrements. This is supported by evidence obtained in an earlier study in which it was found that even larger concrements in the gallbladder did not prevent the regression of the inflammatory reaction as soon as dihydrocholesterol was removed from the diet.[2]

The experiments reported here favor the view that the development of cholelithiasis and cholecystitis in dihydrocholesterol-fed animals is due, at least in part, to the conversion of this sterol into abnormal bile acids (possible 5-cholanic acids). These combine with glycine to form bile

salts which differ from the normally occurring compounds. The abnormal bile salts have an irritating effect upon the biliary tract and in addition seem to reduce the solubility of normal biliary constituents. It would be of interest to learn whether 5α-cholanic acids are capable of producing lesions in the biliary tract similar to those found after feeding dihydrocholesterol.

Summary

The early pathogenesis of dihydrocholesterol-induced cholecystitis and cholelithiasis was investigated in the rabbit. Lesions of the biliary tract, consisting of edema, cellular infiltration and concrements, were first seen 7 days after institution of dihydrocholesterol feeding. Under the experimental conditions employed, the inflammatory reaction of the biliary tract tended to precede the appearance of the biliary concrements. The gallstones consisted largely of glycine conjugates of cholanic acids derived about equally from cholesterol and dihydrocholesterol. It is suggested that the formation of these gallstones is due in part to the presence of allocholanic acids derived from dihydrocholesterol.

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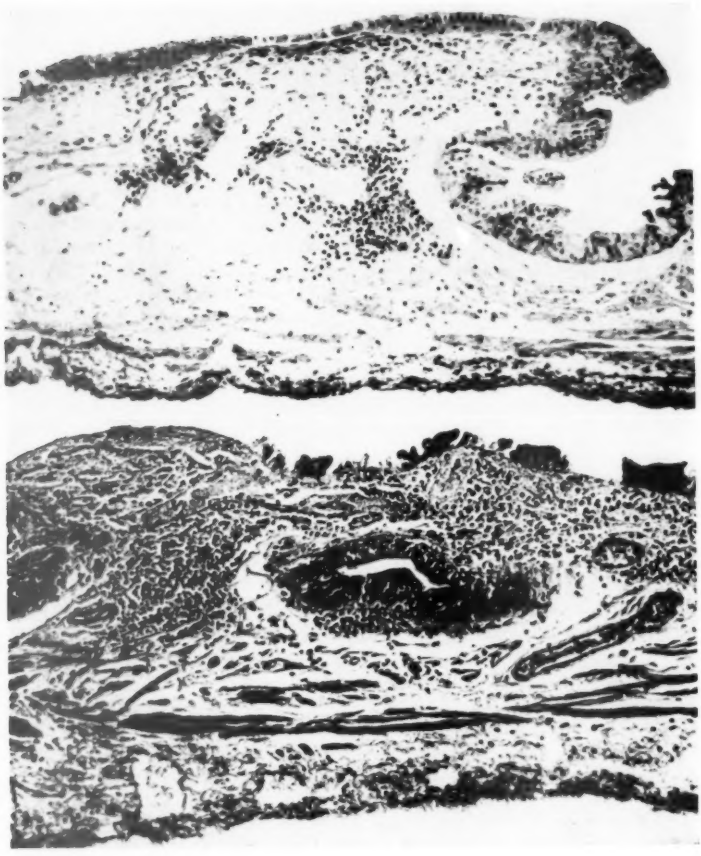
[*Illustrations follow*]

Legends for Figures

Sections were stained with hematoxylin and eosin.

Fig. 1. Gallbladder of a rabbit fed 0.5 per cent dihydrocholesterol for 7 days. No calculi were present in the gallbladder, yet the lamina propria is edematous and infiltrated with lymphocytes and histiocytes. The infiltration is both diffuse and perivascular but is largely confined to the lamina propria. This lesion was considered a minimal though definite inflammatory reaction. × 120.

Fig. 2. Gallbladder of a rabbit fed 0.5 per cent dihydrocholesterol for 18 days. The calculi in the fundus weighed 168 mg. (wet weight). The gallbladder wall is edematous. The mucosal folds are flattened. The lymphocytic and histiocytic cellular infiltration has greatly increased in severity over that seen in Figure 1 and extends throughout all coats of the wall. × 120.



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